Application
for
United States Letters Patent

To all whom it may concern:

Be it known that we,

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have invented certain new and useful improvements in

DNA ENCODING A HUMAN RECEPTOR (hp15a) AND USES THEREOF

of which the following is a full, clear and exact description.

DNA ENCODING A HUMAN RECEPTOR (hp15a) AND USES THEREOF

BACKGROUND OF THE INVENTION

Throughout this application, various publications are referenced in parentheses by author and year. Full citations for these references may be found at the end of the specification immediately preceding the sequence listings and the claims. The disclosure of these publications in their entireties are hereby incorporated by reference into this application in order to describe more fully the state of the art to which this invention pertains.

Neuroregulators comprise a diverse group of natural products that subserve or modulate communication in the nervous system. They include, but are not limited to, neuropeptides, amino acids, biogenic amines, lipids and lipid metabolites, and other metabolic byproducts. Many of these neuroregulator substances interact with specific cell surface receptors which transduce signals from the outside to the inside of the cell. G-protein coupled receptors (GPCRs) represent a major class of cell surface receptors with which many neurotransmitters interact to mediate their effects. GPCRs are characterized by seven membrane-spanning domains and are coupled to their effectors via G-proteins linking receptor activation with intracellular biochemical sequelae such as stimulation of adenylyl cyclase. While the structural motifs that characterize a GPCR can be recognized in the predicted amino acid sequence of a novel receptor, the endogenous ligand that activates the GPCR cannot necessarily be predicted from its primary structure. Thus, a novel receptor sequence may be designated as an orphan GPCR when its functional identity as a G-protein coupled receptor can be defined but its endogenous activating ligand cannot.

The hp15a receptor is such an orphan GPCR. Isolated from genomic DNA by reduced stringency homology cloning using probes designed from receptors later designated $5HT_{1D3}$ and $5HT_{1A}$, the hp15a receptor gene encodes a novel GPCR of unknown function. Its closest relatives are other GPCRs, but none exhibits greater than 26% amino acid identity with hp15a. This level of identity is typically too low to permit predictions with respect to activating ligands. However, the endogenous ligand for the hp15a receptor is likely to be a neuromodulator since the hp15a receptor is present in several regions of the human brain.

Using a homology screening approach to clone new receptor genes, we describe here the isolation and characterization of a clone encoding a novel receptor. We have designated the clone the hp15a receptor gene Use of the receptor encoded by the hp15a receptor gene enables the discovery of the endogenous activating ligand which is a potentially important neuroregulator. It further enables elucidation of possible receptor diversity and of the existence of multiple subtypes within a family of receptors of which hp15a is a member. It is contemplated that this receptor will serve as a valuable tool for designing drugs for treating various pathophysiological conditions such as memory loss, depression, anxiety, epilepsy, pain, hypertension, locomotor problems, circadian rhythm disorders, eating/body weight disorders, sexual/reproductive disorders, nasal congestion, diarrhea, and gastrointestinal and cardiovascular disorders.

SUMMARY OF THE INVENTION

This invention provides an isolated nucleic acid

encoding a mammalian hp15a receptor. In one embodiment, the mammalian hp15a receptor is a human hp15a receptor.

5   This invention further provides an isolated nucleic acid encoding a human hp15a receptor analog, a vector comprising a nucleic acid encoding a mammalian hp15a receptor, e.g. a human hp15a receptor, particularly vector adapted for expression of a hp15a receptor in

10 mammalian or non-mammalian cells. One such vector which expresses the human hp15a receptor is a plasmid designated hp15a (ATCC Accession No. 209447).

This invention also provides a purified mammalian hp15a

15 receptor protein.

In addition, this invention provides a cell comprising a vector which comprises a nucleic acid encoding a mammalian hp15a receptor and a membrane preparation

20 isolated from such a cell.

This invention further provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a

25 mammalian hp15a receptor, wherein the probe has a unique sequence corresponding to a sequence present within one of the two strands of the nucleic acid encoding the mammalian hp15a receptor that is contained in plasmid hp15a (ATCC Accession No. 209447).

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This invention further provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian hp15a receptor, wherein the probe has a

35 unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 1 (Seq. I.D. No. 1) or (b) the reverse complement

thereto.

This invention also provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides which is complementary to a unique segment of the sequence of a nucleic acid molecule encoding a mammalian hp15a receptor.

This invention provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides which is complementary to the antisense sequence of a unique segment of the sequence of a nucleic acid molecule encoding a mammalian hp15a receptor.

Further, this invention provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to RNA encoding a mammalian hp15a receptor, so as to prevent translation of the RNA. This invention also provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to genomic DNA encoding a mammalian hp15a receptor.

This invention further provides an antibody capable of binding to a mammalian hp15a receptor. This invention also provides an agent capable of competitively inhibiting the binding of the antibody to a mammalian hp15a receptor.

This invention provides a pharmaceutical composition comprising (a) an amount of the oligonucleotide described above capable of passing through a cell membrane and effective to reduce expression of a mammalian hp15a receptor and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane.

Still further, this invention provides a transgenic, nonhuman mammal expressing DNA encoding a mammalian hp15a receptor. This invention also provides a transgenic, nonhuman mammal comprising a homologous recombination knockout of the native mammalian hp15a receptor. This invention further provides a transgenic, nonhuman mammal whose genome comprises antisense DNA complementary to the DNA encoding a mammalian hp15a receptor so placed within the genome as to be transcribed into antisense mRNA which is complementary to mRNA encoding the mammalian hp15a receptor and which hybridizes to mRNA encoding the mammalian hp15a receptor, thereby reducing its translation.

Importantly, this invention provides a process for identifying a chemical compound which specifically binds to a mammalian hp15a receptor which comprises contacting cells containing DNA encoding and expressing on their cell surface the mammalian hp15a receptor, wherein such cells do not normally express the mammalian hp15a receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the mammalian hp15a receptor.

Alternatively or additionally, this invention provides a process for identifying a chemical compound which specifically binds to a mammalian hp15a receptor which comprises contacting a membrane fraction from a cell extract of cells containing DNA encoding and expressing on their cell surface the mammalian hp15a receptor, wherein such cells do not normally express the mammalian hp15a receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the mammalian hp15a receptor.

Furthermore, this invention provides a process involving competitive binding for identifying a chemical compound which specifically binds to a mammalian hp15a receptor which comprises contacting cells expressing on their cell surface the mammalian hp15a receptor, wherein such cells do not normally express the mammalian hp15a receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and separately with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the mammalian hp15a receptor, a decrease in the binding of the second chemical compound to the mammalian hp15a receptor in the presence of the chemical compound indicating that the chemical compound binds to the mammalian hp15a receptor.

This invention also provides a process involving competitive binding for identifying a chemical compound which specifically binds to a mammalian hp15a receptor which comprises contacting a membrane fraction from a cell extract of cells expressing on their cell surface the mammalian hp15a receptor, wherein such cells do not normally express the mammalian hp15a receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and separately with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the mammalian hp15a receptor, a decrease in the binding of the second chemical compound to the mammalian hp15a receptor in the presence of the chemical compound indicating that the chemical compound binds to the mammalian hp15a receptor.

This invention further provides a method of screening

a plurality of chemical compounds not known to bind to a mammalian hp15a receptor to identify a compound which specifically binds to the mammalian hp15a receptor, which comprises (a) contacting cells transfected with and expressing DNA encoding the mammalian hp15a receptor with a compound known to bind specifically to the mammalian hp15a receptor under condition permitting binding of the compound known to bind; (b) contacting the cells resulting from step (a) with the plurality of compounds not known to bind specifically to the mammalian hp15a receptor, under conditions permitting binding of compounds known to bind the hp15a receptor; (c) determining whether the binding of the compound known to bind to the mammalian hp15a receptor is reduced in the presence of one or more compound within the plurality of compounds, relative to the binding of the compound in the absence of such one or more compound within the plurality of compounds; and if so (d) separately determining the binding to the mammalian hp15a receptor of such one or more compound included in the plurality of compounds, so as to thereby identify such one or more compound which specifically binds to the mammalian hp15a receptor.

This invention provides a method of screening a plurality of chemical compounds not known to bind to a mammalian hp15a receptor to identify a compound which specifically binds to the mammalian hp15a receptor, which comprises (a) contacting a membrane fraction from cells transfected with and expressing DNA encoding the mammalian hp15a receptor with a compound known to bind specifically to the mammalian hp15a receptor under conditions permitting binding of the compound known to bind; (b) contacting the membrane fraction resulting from step (a) with the plurality of compounds not known to bind specifically to the mammalian hp15a receptor, under conditions permitting binding of compounds known

to bind the mammalian hp15a receptor; (c) determining whether the binding of the compound known to bind to the mammalian hp15a receptor is reduced in the presence of one or more compound within the plurality of compounds, relative to the binding of such one or more compound in the absence of the plurality of compounds; and if so (d) separately determining the binding to the mammalian hp15a receptor of such one or more compound included in the plurality of compounds, so as to thereby identify such one or more compound which specifically binds to the mammalian hp15a receptor.

This invention provides a method of detecting expression of a mammalian hp15a receptor by detecting the presence of mRNA coding for the mammalian hp15a receptor which comprises obtaining total mRNA from the cell and contacting the mRNA so obtained with a nucleic acid probe under hybridizing conditions, detecting the presence of mRNA hybridizing to the probe, and thereby detecting the expression of the mammalian hp15a receptor by the cell.

This invention provides a method of detecting the presence of a mammalian hp15a receptor on the surface of a cell which comprises contacting the cell with an antibody under conditions permitting binding of the antibody to the receptor, detecting the presence of the antibody bound to the cell, and thereby detecting the presence of the mammalian hp15a receptor on the surface of the cell.

This invention provides a method of determining the physiological effects of varying levels of activity of mammalian hp15a receptors which comprises producing a transgenic, nonhuman mammal whose levels of mammalian hp15a receptor activity are varied by use of an inducible promoter which regulates mammalian hp15a

receptor expression.

This invention provides a method of determining the physiological effects of varying levels of activity of mammalian hp15a receptors which comprises producing a panel of transgenic, nonhuman mammals each expressing a different amount of mammalian hp15a receptor.

This invention provides a method for identifying an antagonist capable of alleviating an abnormality wherein the abnormality is alleviated by decreasing the activity of a mammalian hp15a receptor comprising administering a compound to a transgenic, nonhuman mammal as described above and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal as a result of overactivity of a mammalian hp15a receptor, the alleviation of the abnormality identifying the compound as an antagonist. This invention also provides an antagonist identified by this method and a pharmaceutical composition comprising a therapeutically effective amount of an antagonist identified by this method and a pharmaceutically acceptable carrier.

This invention provides a method of treating an abnormality .. a subject wherein the abnormality is alleviated by decreasing the activity of a mammalian hp15a receptor which comprises administering to the subject an effective dose of such a pharmaceutical composition, thereby treating the abnormality.

This invention provides a method for identifying an agonist capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian hp15a receptor comprising administering a compound to a transgenic,

nonhuman mammal, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal, the alleviation of the abnormality identifying the compound as an agonist. This invention also provides an agonist identified by this method and a pharmaceutical composition comprising a therapeutically effective amount of an agonist identified by this method and a pharmaceutically acceptable carrier. This invention provides a method of treating an normality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian hp15a receptor which comprises administering to the subject an effective dose of such a pharmaceutical composition, thereby treating the abnormality.

In addition, this invention provides a method for diagnosing a predisposition to a disorder associated with the activity of a specific mammalian allele which comprises: (a) obtaining DNA of subjects suffering from the disorder; (b) performing a restriction digest of the DNA with a panel of restriction enzymes; (c) electrophoretically separating the resulting DNA fragments on a sizing gel; (d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a mammalian hp15a receptor and labeled with a detectable marker; (e) detecting labeled bands· which have hybridized to the DNA encoding a mammalian hp15a receptor labeled with a detectable marker to create a unique band pattern specific to the DNA of subjects suffering from the disorder; (f) preparing DNA obtained for diagnosis by steps (a)-(e); and (g) comparing the unique band pattern specific to the DNA of subjects suffering from the disorder from step (e) and the DNA obtained for diagnosis from step (f) to determine

whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same.

5   This invention also provides a method of preparing a purified mammalian hp15a receptor which comprises: (a) inducing cells to express the mammalian hp15a receptor; (b) recovering the mammalian hp15a receptor from the induced cells; and (c) purifying the mammalian hp15a
10   receptor so recovered.

This invention further provides a method of preparing a purified mammalian hp15a receptor which comprises: inserting a nucleic acid encoding the mammalian hp15a
15   receptor into a suitable vector; (b) introducing the resulting vector in a suitable host cell; (c) placing the resulting cell in suitable condition permitting the production of the mammalian hp15a receptor; (d) recovering the mammalian hp15a receptor produced by the
20   resulting cell; and (e) purifying and isolating the mammalian hp15a receptor so recovered.

This invention provides a process for determining whether a chemical compound is a mammalian hp15a
25   receptor agonist which comprises contacting cells transfected with and expressing DNA encoding the mammalian hp15a receptor with the compound under conditions permitting the activation of the mammalian hp15a receptor, and detecting an increase in mammalian
30   hp15a receptor activity, so as to thereby determine whether the compound is a mammalian hp15a receptor agonist. This invention also provides a pharmaceutical composition which comprises an amount of a mammalian hp15a receptor agonist determined by this process
35   effective to increase activity of a mammalian hp15a receptor and a pharmaceutically acceptable carrier.

This invention provides a process for determining whether a chemical compound is a mammalian hp15a receptor antagonist which comprises contacting cells transfected with and expressing DNA encoding the mammalian hp15a receptor with the compound in the presence of a known mammalian hp15a receptor agonist, under conditions permitting the activation of the mammalian hp15a receptor, and detecting a decrease in mammalian hp15a receptor activity, so as to thereby determine whether the compound is a mammalian hp15a receptor antagonist. This invention also provides a pharmaceutical composition which comprises an amount of a mammalian hp15a receptor antagonist determined by this process effective to reduce activity of a mammalian hp15a receptor and a pharmaceutically acceptable carrier.

This invention provides a process for determining whether a chemical compound specifically binds to and activates a mammalian hp15a receptor, which comprises contacting cells producing a second messenger response and expressing on their cell surface the mammalian hp15a receptor, wherein such cells do not normally express the mammalian hp15a receptor, with the chemical compound under conditions suitable for activation of the mammalian hp15a receptor, and measuring the second messenger response in the presence and in the absence of the chemical compound, a change in the second messenger response in the presence of the chemical compound indicating that the compound activates the mammalian hp15a receptor. This invention also provides a compound determined by this process. This invention further provides a pharmaceutical composition which comprises an amount of the compound (a hp15a receptor agonist) determined by this process effective to increase activity of a mammalian hp15a receptor and a pharmaceutically acceptable carrier.

This invention provides a process for determining whether a chemical compound specifically binds to and inhibits activation of a mammalian hp15a receptor, which comprises contacting cells producing a second messenger response and expressing on their cell surface the mammalian hp15a receptor, wherein such cells do not normally express the mammalian hp15a receptor, with both the chemical compound and a second chemical compound known to activate the mammalian hp15a receptor, and separately with only the second chemical compound, under conditions suitable for activation of the mammalian hp15a receptor, and measuring the second messenger response in the presence of only the second chemical compound and in the presence of both the second chemical compound and the chemical compound, a smaller change in the second messenger response in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound indicating that the chemical compound inhibits activation of the mammalian hp15a receptor. This invention also provides a compound determined by this process. This invention further provides a pharmaceutical composition which comprises an amount of the compound (a mammalian hp15a receptor antagonist) determined by this effective to reduce activity of a mammalian hp15a receptor and a pharmaceutically acceptable carrier.

This invention provides a method of screening a plurality of chemical compounds not known to activate a mammalian hp15a receptor to identify a compound which activates the mammalian hp15a receptor which comprises: (a) contacting cells transfected with and expressing the mammalian hp15a receptor with the plurality of compounds not known to activate the mammalian hp15a receptor, under conditions permitting activation of the mammalian hp15a receptor; (b) determining whether the

activity of the mammalian hp15a receptor is increased in the presence of one or more of the compounds; and if so (c) separately determining whether the activation of the mammalian hp15a receptor is increased by such compound included in the plurality of compounds, so as to thereby identify such compound which activates the mammalian hp15a receptor. This invention also provides a compound identified by this method. This invention further provides a pharmaceutical composition which comprises an amount of the compound (a mammalian hp15a receptor agonist) identified by this method effective to increase activity of a mammalian hp15a receptor and a pharmaceutically acceptable carrier.

This invention provides a method of screening a plurality of chemical compounds not known to inhibit the activation of a mammalian hp15a receptor to identify a compound which inhibits the activation of the mammalian hp15a receptor, which comprises: (a) contacting cells transfected with and expressing the mammalian hp15a receptor with the plurality of compounds in the presence of a known mammalian hp15a receptor agonist, under conditions permitting activation of the mammalian hp15a receptor; (b) determining whether the activation of the mammalian hp15a receptor is reduced in the presence of one or more compound within the plurality of compounds, relative to the activation of the mammalian hp15a receptor in the absence of such compound within the plurality of compounds; and if so (c) separately determining the inhibition of activation of the mammalian hp15a receptor for such compound included in the plurality of compounds, so as to thereby identify such compound which inhibits the activation of the mammalian hp15a receptor. This invention also provides a compound identified by this method. This invention further provides a pharmaceutical composition which

comprises an amount of the compound (a mammalian hp15a receptor antagonist) identified by this process effective to decrease activity of a mammalian hp15a receptor and a pharmaceutically acceptable carrier.

This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian hp15a receptor which comprises administering to the subject an amount of a compound which is a mammalian hp15a receptor agonist effective to treat the abnormality.

A method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a mammalian hp15a receptor which comprises administering to the subject an amount of a compound which is a mammalian hp15a receptor antagonist effective to treat the abnormality.

This invention further provides a process for making a composition of matter which specifically binds to a mammalian hp15a receptor which comprises identifying a chemical compound using any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a mammalian hp15a receptor and then synthesizing the chemical compound or a novel structure and functional analog or homolog thereof. This invention further provides a process for preparing a pharmaceutical composition which comprises admixing a pharmaceutically acceptable carrier and a pharmaceutically acceptable amount of chemical compound identified by any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a mammalian hp15a receptor or a novel structural and functional analog or homolog thereof.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1B

Nucleotide sequence encoding a human receptor (hp15a) (Seq. I.D. No. 1). In addition, partial 5' and 3' untranslated sequences are shown. The start (ATG) codon (at positions 61-63) and the stop (TAG) codon (at positions 1249-1251) are underlined.

Figures 2A-2C

Deduced amino acid sequence (Seq. I.D. No. 2) of the human receptor (hp15a) encoded by the nucleotide sequence shown Figures 1A-1B (Seq. I.D. No. 1). Seven solid lines designated I-VII located above portions of the sequence indicate the seven putative transmembrane (TM) regions.

Figure 3

Autoradiograph demonstrating hybridization of radiolabeled hp15a probe to RNA extracted from human tissue in a solution hybridization/nuclease protection assay using $\alpha^{32}P$ labeled riboprobe. 2 μg of mRNA were used in each assay. The single band represents mRNA coding for the hp15a receptor extracted from the indicated tissue. Highest levels of mRNA coding for the hp15a are found in: placenta, fetal liver, fetal lung, fetal kidney, lung, and spinal cord. Integrity of RNA was assessed using hybridization to mRNA coding to GAPDH.

DETAILED DESCRIPTION OF THE INVENTION

Throughout this application, the following standard abbreviations are used to indicate specific nucleotide bases:

A = adenine

G = guanine

C = cytosine

T = thymine

U = uracil

M = adenine or cytosine

R = adenine or guanine

W = adenine, thymine, or uracil

S = cytosine or guanine

Y = cytosine, thymine, or uracil

K = guanine, thymine, or uracil

V = adenine, cytosine, or guanine (not thymine or uracil

H = adenine, cytosine, thymine, or uracil (not guanine)

D = adenine, guanine, thymine, or uracil (not cytosine)

B = cytosine, guanine, thymine, or uracil (not adenine)

N = adenine, cytosine, guanine, thymine, or uracil (or other modified base such as inosine)

I = inosine

Furthermore, the term agonist is used throughout this application to indicate any peptide or non-peptidyl compound which increases the activity of any of the polypeptide receptors of the subject invention. The term antagonist is used throughout this application to indicate any peptide or non-peptidyl compound which decreases the activity of any of the polypeptide

receptors of the subject invention.

The activity of a G-protein coupled receptor such as the polypeptides disclosed herein may be measured using any of a variety of functional assays in which activation of the receptor in question results in an observable change in the level of some second messenger system, including but not limited to adenylate cyclase, calcium mobilization, arachidonic acid release, ion channel activity, isositol phospholipid hydrolysis or guanylyl cyclase. Heterologous expression systems utilizing appropriate host cells to express the nucleic acid of the subject invention are used to obtain the desired second messenger coupling. Receptor activity may also be assayed in an oocyte expression system.

It is possible that the mammalian hp15a receptor gene contains introns and furthermore, the possibility exists that additional introns could exist in coding or non-coding regions. In addition, spliced form(s) of mRNA may encode additional amino acids either upstream of the currently defined starting methionine or within the coding region. Further, the existence and use of alternative exons is possible, whereby the mRNA may encode different amino acids within the region comprising the exon. In addition, single amino acid substitutions may arise via the mechanism of RNA editing such that the amino acid sequence of the expressed protein is different than that encoded by the original gene. (Burns et al., 1996; Chu et al., 1996). Such variants may exhibit pharmacologic properties differing from the polypeptide encoded by the original gene.

This invention provides a splice variant of the mammalian hp15a receptor disclosed herein. This invention further provides for alternate translation

initiation sites and alternately spliced or edited variants of nucleic acids encoding mammalian hp15a receptors of this invention.

5   The nucleic acids of the subject invention also include nucleic acid analogs of the human hp15a receptor gene, wherein the human hp15a receptor gene comprises the nucleic acid sequence shown in Fig. 1A-1B or contained in plasmid hp15a (ATCC Accession No. 209447). A

10   nucleic acid analog of the human hp15a receptor gene differs from the human hp15a receptor gene described above in terms of the identity or location of one or more nucleic acid bases (deletion analogs containing less than all of the nucleic acid bases shown in Fig.

15   1A-1B or contained in plasmid hp15a (ATCC Accession No. 209447), substitution analogs wherein one or more nucleic acid bases shown in Fig. 1A-1B or contained in plasmid hp15a (ATCC Accession No. 209447) are replaced by other nucleic acid bases, and addition analogs,

20   wherein one or more nucleic acid bases are added to a terminal or medial portion of the nucleic acid sequence) and which encode proteins which share some or all of the properties of the protein encoded by the nucleic acid sequence shown in Fig. 1A-1B or contained

25   in plasmid hp15a (ATCC Accession No. 209447). In one embodiment of the present invention, the nucleic acid analog encodes a protein which has an amino acid sequence identical to that shown in Fig. 2A-2C or encoded by the nucleic acid sequence contained in

30   plasmid hp15a (ATCC Accession No. 209447). In another embodiment, the nucleic acid analog encodes a protein having an amino acid sequence which differs from the amino acid sequence shown in Fig. 2A-2C or encoded by the nucleic acid contained in plasmid hp15a (ATCC

35   Accession No. 209447). In a further embodiment, the protein encoded by the nucleic acid analog has a function which is the same as the function of the

receptor protein having the amino acid sequence shown in Fig. 2A-2C. In another embodiment, the function of the protein encoded by the nucleic acid analog differs from the function of the receptor protein having the amino acid sequence shown in Fig. 2A-2C. In separate embodiments, the variation in the nucleic acid sequence is less than 20 number of base pairs; preferably, less than 10 number of base pairs; more preferably, less than 5 number of base pairs. In another embodiment, the variation in the nucleic acid sequence occurs only within the transmembrane (TM) regions of the protein. In a further embodiment, the variation in the nucleic acid sequence occurs only outside of the TM regions.

This invention provides the above-described isolated nucleic acid, wherein the nucleic acid is DNA. In an embodiment, the DNA is cDNA. In another embodiment, the DNA is genomic DNA. In still another embodiment, the nucleic acid is RNA. Methods for production and manipulation of nucleic acid molecules are well known in the art.

This invention further provides nucleic acid which is degenerate with respect to the DNA corresponding to the hp15a coding sequence within the plasmid hp15a (ATCC Accession No. 209447).

This invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ.from those of the polypeptides of this invention, but which do not produce phenotypic changes. Alternately or additionally, this invention also encompasses DNAs, cDNAs, and RNAs which hybridize to the DNA, cDNA, and RNA of the subject invention. Hybridization methods are well known to those of skill in the art.

The nucleic acids of the subject invention also include

nucleic acid molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs wherein one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These molecules include: the incorporation of codons preferred for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors. The creation of polypeptide analogs is well known to those of skill in the art (R.F. Spurney et al. (1997); Fong, T.M. et al. (1995); Underwood, D.J. et al. (1994); Graziano, M.P. et al. (1996); Guam X.M. et al. (1995)).

The modified polypeptides of this invention may be transfected into cells either transiently or stably using methods well-known in the art, examples of which are disclosed herein. This invention also provides for binding assays using the modified polypeptides, in which the polypeptide is expressed either transiently or in stable cell lines. This invention further provides a compound identified using a modified polypeptide in a binding assay such as the binding assays described herein.

The nucleic acids described and claimed herein are useful as products for the large scale synthesis of the polypeptides by a variety of recombinant techniques.

The nucleic acid molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide and related products.

This invention provides an isolated nucleic acid encoding a mammalian hp15a receptor. In one embodiment, the nucleic acid is DNA. In another embodiment, the DNA is cDNA. In another embodiment, the DNA is genomic DNA. In another embodiment, the nucleic acid is RNA.

This invention further provides an isolated nucleic acid encoding a human hp15a receptor analog.

In one embodiment of the present invention, the mammalian hp15a receptor is a human hp15a receptor.

This invention also provides an isolated nucleic acid encoding a species homolog of the human hp15a receptor. In one embodiment, the nucleic acid encodes a mammalian hp15a receptor homolog which has substantially the same amino acid sequence as does the human hp15a receptor encoded by the plasmid hp15a (ATCC Accession No. 209447). In another embodiment, the nucleic acid encodes a mammalian hp15a receptor homolog which has about 65% amino acid identity to the human hp15a receptor encoded by the plasmid hp15a (ATCC Accession No. 209447). In a further embodiment, the nucleic acid encodes a mammalian hp15a receptor which has about 75% amino acid identity to the human hp15a receptor encoded by the plasmid hp15a (ATCC Accession No. 209447). In another embodiment, the nucleic acid encodes a mammalian hp15a receptor which has about 85% amino acid identity to the human hp15a receptor encoded by the

plasmid hp15a (ATCC Accession No. 209447). In a further embodiment, the nucleic acid encodes a mammalian hp15a receptor which has about 95% amino acid identity to the human hp15a receptor encoded by the plasmid hp15a (ATCC Accession No. 209447). In a further embodiment, the nucleic acid encodes a mammalian hp15a receptor homolog which has an amino acid sequence identical to that of the human hp15a receptor encoded by the plasmid hp15a (ATCC Accession No. 209447). In another embodiment, the mammalian hp15a receptor homolog has about 70% nucleic acid identity to the human hp15a receptor gene contained in plasmid hp15a (ATCC Accession No. 209447). In a further embodiment, the mammalian hp15a receptor homolog has about 80% nucleic acid identity to the human hp15a receptor gene contained in the plasmid hp15a (ATCC Accession No. 209447). In another embodiment, the mammalian hp15a receptor homolog has about 90% nucleic acid identity to the human hp15a receptor gene contained in the plasmid hp15a (ATCC Accession No. 209447). In a further embodiment, the mammalian hp15a receptor homolog has about 100% nucleic acid identity to the human hp15a receptor gene contained in the plasmid hp15a (ATCC Accession No. 209447). Examples of methods for isolating and purifying species homologs have been described elsewhere (U.S. Patent No. 5,602,024)

In another embodiment, the nucleic acid encodes a human hp15a receptor which has an amino acid sequence identical to that encoded by the plasmid hp15a (ATCC Accession No. 209447). In a further embodiment, the human hp15a receptor has a sequence substantially the same as the amino acid sequence shown in Figure 2A-2C (Seq. I.D. No. 2). In another embodiment, the human hp15a receptor has an amino acid sequence identical to the amino acid sequence shown in Figure 2A-2C (Seq.

I.D. No. 2).

This invention provides an isolated nucleic acid encoding a modified mammalian hp15a receptor, which differs from a mammalian hp15a receptor by having an amino acid(s) deletion, replacement, or addition in the third intracellular domain. In one embodiment, the modified mammalian hp15a receptor is a human hp15a receptor.

This invention provides a purified mammalian hp15a receptor protein. In one embodiment, the purified mammalian hp15a receptor protein is a human hp15a receptor protein.

This invention provides a vector comprising the nucleic acid encoding a mammalian hp15a receptor. In another embodiment, the mammalian hp15a receptor is a human hp15a receptor.

In an embodiment, the vector is adapted for expression in a bacterial cell which comprises the regulatory elements necessary for expression of the nucleic acid in the bacterial cell operatively linked to the nucleic acid encoding the mammalian hp15a receptor as to permit expression thereof. In another embodiment, the vector is adapted for expression in an amphibian cell which comprises the regulatory elements necessary for expression of the nucleic acid in the amphibian cell operatively linked to the nucleic acid encoding the mammalian hp15a receptor as to permit expression thereof. In a further embodiment, the vector is adapted for expression in a yeast cell which comprises the regulatory elements necessary for expression of the nucleic acid in the yeast cell operatively linked to the nucleic acid encoding the mammalian hp15a receptor so as to permit expression thereof. In an embodiment,

the vector is adapted for expression in an insect cell which comprises the regulatory elements necessary for expression of the nucleic acid in the insect cell operatively linked to the nucleic acid encoding the mammalian hp15a receptor so as to permit expression thereof. In another embodiment, the vector is a baculovirus. In a further embodiment, the vector is adapted for expression in a mammalian cell which comprises the regulatory elements necessary for expression of the nucleic acid in the mammalian cell operatively linked to the nucleic acid encoding the mammalian hp15a receptor so as to permit expression thereof. In one embodiment, the vector is a plasmid.

This invention provides a plasmid designated hp15a (ATCC Accession No. 209447). This plasmid comprises the regulatory elements necessary for expression of DNA in a mammalian cell operatively linked to DNA encoding the mammalian hp15a receptor so as to permit expression thereof.

This plasmid (hp15a) was deposited on November 11, 1997, with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and was accorded ATCC Accession No. 209447.

This invention further provides vector or plasmid which comprises modified untranslated sequences, which are beneficial for expression in desired host cells or for use in binding or functional assays. For example, a vector or plasmid with untranslated sequences of varying lengths may express differing amounts of the polypeptide depending upon the host cell used. In an embodiment, the vector or plasmid comprises the coding

sequence of the polypeptide and the regulatory elements necessary for expression in the host cell.

This invention provides a cell comprising a vector comprising a nucleic acid encoding the mammalian hp15a receptor. In an embodiment, the cell is a non-mammalian cell. In a further embodiment, the non-mammalian cell is a Xenopus oocyte cell or a Xenopus melanophore cell. In another embodiment, the cell is a mammalian cell. In a further embodiment, the mammalian cell is a COS-7 cell, a 293 human embryonic kidney cell, a NIH-3T3 cell, a LM(tk-) cell, a mouse Y1 cell, or a CHO cell.

This invention provides an insect cell comprising a vector adapted for expression in an insect cell which comprises a nucleic acid encoding a mammalian hp15a receptor. In another embodiment, the insect cell is an Sf9 cell, an Sf21 cell or a HighFive cell.

This invention provides a membrane preparation isolated from any of the cells described above.

This invention provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian hp15a receptor, wherein the probe has a unique sequence corresponding to a sequence present within one of the two strands of the nucleic acid encoding the mammalian hp15a receptor and are contained in plasmid hp15a (ATCC Accession No. 209447). This invention also provides a nucleic acid probe comprising at least 15 nucleotides, which probe specifically hybridizes with a nucleic acid encoding a mammalian hp15a receptor, wherein the probe has a unique sequence corresponding to a sequence present within (a) the nucleic acid sequence shown in Figure 1 (Seq. I.D. No. 1) or (b) the reverse

complement thereto. In one embodiment, the nucleic acid is DNA. In another embodiment, the nucleic acid is RNA.

This invention provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides which is complementary to a unique fragment of the sequence of a nucleic acid molecule encoding a mammalian hp15a receptor. This invention also provides a nucleic acid probe comprising a nucleic acid molecule of at least 15 nucleotides which is complementary to the antisense sequence of a unique fragment of the sequence of a nucleic acid molecule encoding a mammalian hp15a receptor.

As used herein, the phrase specifically hybridizing means the ability of a nucleic acid molecule to recognize a nucleic acid sequence complementary to its own and to form double-helical segments through hydrogen bonding between complementary base pairs.

Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. DNA probe molecules may be produced by insertion of a DNA molecule which encodes the polypeptides of this invention into suitable vectors, such as plasmids or bacteriophages, followed by transforming into suitable bacterial host cells, replication in the transformed bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from DNA synthesizers.

RNA probes may be generated by inserting the DNA

molecule which encodes the polypeptides of this invention downstream of a bacteriophage promoter such as T3, T7, or SP6. Large amounts of RNA probe may be produced by incubating the labeled nucleotides with the linearized fragment where it contains an upstream promoter in the presence of the appropriate RNA polymerase.

This invention provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to RNA encoding a mammalian hp15a receptor, so as to prevent translation of the RNA. This invention also provides an antisense oligonucleotide having a sequence capable of specifically hybridizing to genomic DNA encoding a mammalian hp15a receptor. In one embodiment, the oligonucleotide comprises chemically modified nucleotides or nucleotide analogues.

This invention provides an antibody capable of binding to a mammalian hp15a receptor encoded by a nucleic acid encoding a mammalian hp15a receptor. In one embodiment, the mammalian hp15a receptor is a human hp15a receptor. This invention also provides an agent capable of competitively inhibiting the binding of the antibody to a mammalian hp15a receptor. In one embodiment, the antibody is a monoclonal antibody or antisera.

This invention provides a pharmaceutical composition comprising (a) an amount of the oligonucleotide capable of passing through a cell membrane and effective to reduce expression of a mammalian hp15a receptor and (b) a pharmaceutically acceptable carrier capable of passing through the cell membrane. In an embodiment, the oligonucleotide is coupled to a substance which inactivates mRNA. In a further embodiment, the substance which inactivates mRNA is a

ribozyme. In another embodiment, the pharmaceutically acceptable carrier comprises a structure which binds to a mammalian hp15a receptor on a cell capable of being taken up by the cells after binding to the structure. In a further embodiment, the pharmaceutically acceptable carrier is capable of binding to a mammalian hp15a receptor which is specific for a selected cell type.

This invention provides a pharmaceutical composition which comprises an amount of an antibody effective to block binding of a ligand to a human hp15a receptor and a pharmaceutically acceptable carrier.

As used herein, the phrase pharmaceutically acceptable carrier means any of the standard pharmaceutically acceptable carriers. Examples include, but are not limited to, phosphate buffered saline, physiological saline, water, and emulsions, such as oil/water emulsions.

This invention provides a transgenic, nonhuman mammal expressing DNA encoding a mammalian hp15a receptor. This invention also provides a transgenic, nonhuman mammal comprising a homologous recombination knockout of the native mammalian hp15a receptor. This invention further provides a transgenic, nonhuman mammal whose genome comprises antisense DNA complementary to the DNA encoding a mammalian hp15a receptor so placed within the genome as to be transcribed into antisense mRNA which is complementary to mRNA encoding the mammalian hp15a receptor and which hybridizes to mRNA encoding the mammalian hp15a receptor, thereby reducing its translation. In an embodiment, the DNA encoding the mammalian hp15a receptor additionally comprises an inducible promoter. In another embodiment, the DNA encoding the mammalian hp15a receptor additionally

comprises tissue specific regulatory elements. In a further embodiment, the transgenic, nonhuman mammal is a mouse.

5   Animal model systems which elucidate the physiological and behavioral roles of the polypeptides of this invention are produced by creating transgenic animals in which the activity of the polypeptide is either increased or decreased, or the amino acid sequence of 10   the expressed polypeptide is altered, by a variety of techniques. Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA encoding the polypeptide, by microinjection, electroporation, retroviral 15   transfection or other means well known to those in the art, into appropriate fertilized embryos in order to produce a transgenic animal or 2) Homologous recombination of mutant or normal, human or animal versions of these genes with the native gene locus in 20   transgenic animals to alter the regulation of expression or the structure of these polypeptide sequences. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing 25   an animal that cannot express native polypeptides but does express, for example, an inserted mutant polypeptide, which has replaced the native polypeptide in the animal's genome by recombination, resulting in underexpression of the receptor. Microinjection adds 30   genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added polypeptides, resulting in overexpression of the polypeptides.

35   One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice are mated, and the resulting fertilized eggs are

dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium. DNA or cDNA encoding a polypeptide of this invention is purified from a vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the trans-gene. Alternatively, or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

This invention provides a process for identifying a chemical compound which specifically binds to a mammalian hp15a receptor which comprises contacting cells containing DNA encoding and expressing on their cell surface the mammalian hp15a receptor, wherein such cells do not normally express the mammalian hp15a receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the mammalian hp15a receptor. This invention also provides a process for identifying a chemical compound which specifically binds to a mammalian hp15a receptor which comprises contacting a membrane fraction from a cell extract of cells

containing DNA encoding and expressing on their cell surface the mammalian hp15a receptor, wherein such cells do not normally express the mammalian hp15a receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the mammalian hp15a receptor. In one embodiment, the mammalian hp15a receptor is a human hp15a receptor. In another embodiment, the mammalian hp15a receptor has substantially the same amino acid sequence as the mammalian hp15a receptor encoded by plasmid hp15a (ATCC Accession No. 209447). In a further embodiment, the mammalian hp15a receptor has substantially the same amino acid sequence as that shown in Figure 2A-2C (Seq. I.D. No. 2). In another embodiment, the mammalian hp15a receptor has the amino acid sequence shown in Figure 2A-2C (Seq. I.D. No. 2). In one embodiment, the compound is not previously known to bind to a mammalian hp15a receptor. This invention further provides a compound identified by the above-described process.

In one embodiment of the above-described processes, the cell is an insect cell. In another embodiment, the cell is a mammalian cell. In a further embodiment, the cell is nonneuronal in origin. In a further embodiment, the nonneuronal cell is a COS-7 cell, 293 human embryonic kidney cell, a CHO cell, a NIH-3T3 cell, a mouse Y1 cell, or a LM(tk-) cell. In an embodiment, the compound is a compound not previously known to bind to a mammalian hp15a receptor. This invention also provides a compound identified by the above-described process.

This invention provides a process involving competitive binding for identifying a chemical compound which specifically binds to a mammalian hp15a receptor which comprises separately contacting cells expressing on

their cell surface the mammalian hp15a receptor, wherein such cells do not normally express the mammalian hp15a receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the mammalian hp15a receptor, a decrease in the binding of the second chemical compound to the mammalian hp15a receptor in the presence of the chemical compound indicating that the chemical compound binds to the mammalian hp15a receptor.

This invention also provides a process involving competitive binding for identifying a chemical compound which specifically binds to a mammalian hp15a receptor which comprises separately contacting a membrane fraction from a cell extract of cells expressing on their cell surface the mammalian hp15a receptor, wherein such cells do not normally express the mammalian hp15a receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the mammalian hp15a receptor, a decrease in the binding of the second chemical compound to the mammalian hp15a receptor in the presence of the chemical compound indicating that the chemical compound binds to the mammalian hp15a receptor.

In one embodiment, the mammalian hp15a receptor is a human hp15a receptor. In another embodiment, the human hp15a receptor has substantially the same amino acid sequence as the human hp15a receptor encoded by plasmid hp15a (ATCC Accession No. 209447). In a further embodiment, the mammalian hp15a receptor has

substantially the same amino acid sequence as that shown in Figure 2A-2C (Seq. I.D. No. 2). In another embodiment, the mammalian hp15a receptor has the amino acid sequence shown in Figure 2A-2C (Seq. I.D. No. 2).

In one embodiment, the cell is an insect cell. In another embodiment, the cell is a mammalian cell. In a further embodiment, the cell is nonneuronal in origin. In another embodiment, the nonneuronal cell is a COS-7 cell, 293 human embryonic kidney cell, a CHO cell, a NIH-3T3 cell, a mouse Y1 cell, or a LM(tk-) cell. In one embodiment, the compound is not previously known to bind to a mammalian hp15a receptor.

This invention provides a compound identified by the above-described process.

This invention provides a method of screening a plurality of chemical compounds not known to bind to a mammalian hp15a receptor to identify a compound which specifically binds to the mammalian hp15a receptor, which comprises (a) contacting cells transfected with and expressing DNA encoding the mammalian hp15a receptor with a compound known to bind specifically to the mammalian hp15a receptor; (b) contacting the preparation of step (a) with the plurality of compounds not known to bind specifically to the mammalian hp15a receptor, under conditions permitting binding of compounds known to bind the mammalian hp15a receptor; (c) determining whether the binding of the compound known to bind to the mammalian hp15a receptor is reduced in the presence of the compounds within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if so (d) separately determining the binding to the mammalian hp15a receptor of compounds included in the plurality of compounds, so as to thereby identify the

compound which specifically binds to the mammalian hp15a receptor.

This invention provides a method of screening a plurality of chemical compounds not known to bind to a mammalian hp15a receptor to identify a compound which specifically binds to the mammalian hp15a receptor, which comprises (a) preparing a cell extract from cells transfected with and expressing DNA encoding the mammalian hp15a receptor, isolating a membrane fraction from the cell extract, contacting the membrane fraction with a compound known to bind specifically to the mammalian hp15a receptor; (b) contacting the preparation of step (a) with the plurality of compounds not known to bind specifically to the mammalian hp15a receptor, under conditions permitting binding of compounds known to bind the mammalian hp15a receptor; (c) determining whether the binding of the compound known to bind to the mammalian hp15a receptor is reduced in the presence of the compounds within the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds; and if so (d) separately determining the binding to the mammalian hp15a receptor of compounds included in the plurality of compounds, so as to thereby identify the compound which specifically binds to the mammalian hp15a receptor.

In one embodiment of the above-described methods, the mammalian hp15a receptor is a human hp15a receptor. In another embodiment, the cell is a mammalian cell. In a further embodiment, the mammalian cell is non-neuronal in origin. In another embodiment, the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell, a CHO cell, a mouse Y1 cell, or an NIH-3T3 cell.

This invention also provides a method of detecting expression of a mammalian hp15a receptor by detecting the presence of mRNA coding for the mammalian hp15a receptor which comprises obtaining total mRNA from the cell and contacting the mRNA so obtained from a nucleic acid probe under hybridizing conditions, detecting the presence of mRNA hybridizing to the probe, and thereby detecting the expression of the mammalian hp15a receptor by the cell.

This invention further provides a method of detecting the presence of a mammalian hp15a receptor on the surface of a cell which comprises contacting the cell with an antibody under conditions permitting binding of the antibody to the receptor, detecting the presence of the antibody bound to the cell, and thereby detecting the presence of the mammalian hp15a receptor on the surface of the cell.

This invention provides a method of determining the physiological effects of varying levels of activity of mammalian hp15a receptors which comprises producing a transgenic, nonhuman mammal whose levels of mammalian hp15a receptor activity are varied by use of an inducible promoter which regulates mammalian hp15a receptor expression.

This invention also provides a method of determining the physiological effects of varying levels of activity of mammalian hp15a receptors which comprises producing a panel of transgenic, nonhuman mammals each expressing a different amount of mammalian hp15a receptor.

This invention provides a method for identifying an antagonist capable of alleviating an abnormality wherein the abnormality is alleviated by decreasing the activity of a mammalian hp15a receptor comprising

administering a compound to a transgenic, nonhuman mammal, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal as a result of overactivity of a mammalian hp15a receptor, the alleviation of the abnormality identifying the compound as an antagonist. This invention also provides an antagonist identified by the above-described method. This invention further provides a pharmaceutical composition comprising an antagonist identified by the above-described method and a pharmaceutically acceptable carrier. This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a mammalian hp15a receptor which comprises administering to the subject an effective amount of this pharmaceutical composition, thereby treating the abnormality.

This invention provides a method for identifying an agonist capable of alleviating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian hp15a receptor comprising administering a compound to transgenic, nonhuman mammal, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal, the alleviation of the abnormality identifying the compound as an agonist. This invention also provides an agonist identified by the above-described method. This invention further provides a pharmaceutical composition comprising an agonist identified by the above-described method and a pharmaceutically acceptable carrier. This invention further provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian hp15a receptor which comprises administering to the

subject an effective amount of this pharmaceutical composition, thereby treating the abnormality.

This invention provides a method for diagnosing a predisposition to a disorder associated with the activity of a specific mammalian allele which comprises: (a) obtaining DNA of subjects suffering from the disorder; (b) performing a restriction digest of the DNA with a panel of restriction enzymes; (c) electrophoretically separating the resulting DNA fragments on a sizing gel; (d) contacting the resulting gel with a nucleic acid probe capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule encoding a mammalian hp15a receptor and labeled with a detectable marker; (e) detecting labeled bands which have hybridized to the DNA encoding a mammalian hp15a receptor labeled with a detectable marker to create a unique band pattern specific to the DNA of subjects suffering from the disorder; (f) preparing DNA obtained for diagnosis by steps (a)-(e); and (g) comparing the unique band pattern specific to the DNA of subjects suffering from the disorder from step (e) and the DNA obtained for diagnosis from step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the disorder if the patterns are the same. In one embodiment, a disorder associated with the activity of a specific mammalian allele is diagnosed.

This invention provides a method of preparing the purified mammalian hp15a receptor which comprises: (a) inducing cells to express the mammalian hp15a receptor; (b) recovering the mammalian hp15a receptor from the induced cells; and (c) purifying the mammalian hp15a receptor so recovered.

This invention provides a method of preparing the purified mammalian hp15a receptor which comprises: (a) inserting nucleic acid encoding the mammalian hp15a receptor in a suitable vector; (b) introducing the resulting vector in a suitable host cell; (c) placing the resulting cell in suitable condition permitting the production of the isolated mammalian hp15a receptor; (d) recovering the mammalian hp15a receptor produced by the resulting cell; and (e) purifying the mammalian hp15a receptor so recovered.

This invention provides a process for determining whether a chemical compound is a mammalian hp15a receptor agonist which comprises contacting cells transfected with and expressing DNA encoding the mammalian hp15a receptor with the compound under conditions permitting the activation of the mammalian hp15a receptor, and detecting an increase in mammalian hp15a receptor activity, so as to thereby determine whether the compound is a mammalian hp15a receptor agonist. This invention also provides a process for determining whether a chemical compound is a mammalian hp15a receptor antagonist which comprises contacting cells transfected with and expressing DNA encoding the mammalian hp15a receptor with the compound in the presence of a known mammalian hp15a receptor agonist, under conditions permitting the activation of the mammalian hp15a receptor, and detecting a decrease in mammalian hp15a receptor activity, so as to thereby determine whether the compound is a mammalian hp15a receptor antagonist. In one embodiment, the mammalian hp15a receptor is a human hp15a receptor.

This invention further provides a pharmaceutical composition which comprises an amount of a mammalian hp15a receptor agonist determined by the above-described process effective to increase activity of a

mammalian hp15a receptor and a pharmaceutically acceptable carrier. In one embodiment, the mammalian hp15a receptor agonist is not previously known.

This invention provides a pharmaceutical composition which comprises an amount of a mammalian hp15a receptor antagonist determined by the above-described process effective to reduce activity of a mammalian hp15a receptor and a pharmaceutically acceptable carrier. In one embodiment, the mammalian hp15a receptor antagonist is not previously known.

This invention provides a process for determining whether a chemical compound specifically binds to and activates a mammalian hp15a receptor, which comprises contacting cells producing a second messenger response and expressing on their cell surface the mammalian hp15a receptor, wherein such cells do not normally express the mammalian hp15a receptor, with the chemical compound under conditions suitable for activation of the mammalian hp15a receptor, and measuring the second messenger response in the presence and in the absence of the chemical compound, a change in the second messenger response in the presence of the chemical compound indicating that the compound activates the mammalian hp15a receptor. In one embodiment, the second messenger response comprises chloride channel activation and the change in second messenger is an increase in the level of inward chloride current.

This invention also provides a process for determining whether a chemical compound specifically binds to and inhibits activation of a mammalian hp15a receptor, which comprises separately contacting cells producing a second messenger response and expressing on their cell surface the mammalian hp15a receptor, wherein such cells do not normally express the mammalian hp15a

receptor, with both the chemical compound and a second chemical compound known to activate the mammalian hp15a receptor, and with only the second chemical compound, under conditions suitable for activation of the mammalian hp15a receptor, and measuring the second messenger response in the presence of only the second chemical compound and in the presence of both the second chemical compound and the chemical compound, a smaller change in the second messenger response in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound indicating that the chemical compound inhibits activation of the mammalian hp15a receptor. In one embodiment, the second messenger response comprises chloride channel activation and the change in second messenger response is a smaller increase in the level of inward chloride current in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound.

In one embodiment of the above-described processes, the mammalian hp15a receptor is a human hp15a receptor. In another embodiment, the human hp15a receptor has substantially the same amino acid sequence as encoded by the plasmid hp15a (ATCC Accession No. 209447). In a further embodiment, the human hp15a receptor has substantially the same amino acid sequence as that shown in Figure 2A-2C (Seq. I.D. No. 2). In another embodiment, the human hp15a receptor has an amino acid sequence identical to the amino acid sequence shown in Figure 2A-2C (Seq. I.D. No. 2). In an embodiment, the cell is an insect cell. In a further embodiment, the cell is a mammalian cell. In a still further embodiment, the mammalian cell is nonneuronal in origin. In another embodiment, the nonneuronal cell is a COS-7 cell, CHO cell, 293 human embryonic kidney

cell, NIH-3T3 cell or LM(tk-) cell. In an embodiment, the compound is not previously known to bind to a mammalian hp15a receptor. This invention also provides a compound determined by the above-described processes.

This invention also provides a pharmaceutical composition which comprises an amount of a mammalian hp15a receptor agonist determined by the above-described processes effective to increase activity of a mammalian hp15a receptor and a pharmaceutically acceptable carrier. In one embodiment, the mammalian hp15a receptor agonist is not previously known.

This invention further provides a pharmaceutical composition which comprises an amount of a mammalian hp15a receptor antagonist determined by the above-described processes effective to reduce activity of a mammalian hp15a receptor and a pharmaceutically acceptable carrier. In one embodiment, the mammalian hp15a receptor antagonist is not previously known.

This invention provides a method of screening a plurality of chemical compounds not known to activate a mammalian hp15a receptor to identify a compound which activates the mammalian hp15a receptor which comprises: (a) contacting cells transfected with and expressing the mammalian hp15a receptor with the plurality of compounds not known to activate the mammalian hp15a receptor, under conditions permitting activation of the mammalian hp15a receptor; (b) determining whether the activity of the mammalian hp15a receptor is increased in the presence of the compounds; and if so (c) separately determining whether the activation of the mammalian hp15a receptor is increased by each compound included in the plurality of compounds, so as to thereby identify the compound which activates the mammalian hp15a receptor. In one embodiment, the

mammalian hp15a receptor is a human hp15a receptor.

This invention provides a method of screening a plurality of chemical compounds not known to inhibit the activation of a mammalian hp15a receptor to identify a compound which inhibits the activation of the mammalian hp15a receptor, which comprises: (a) contacting cells transfected with and expressing the mammalian hp15a receptor with the plurality of compounds in the presence of a known mammalian hp15a receptor agonist, under conditions permitting activation of the mammalian hp15a receptor; (b) determining whether the activation of the mammalian hp15a receptor is reduced in the presence of the plurality of compounds, relative to the activation of the mammalian hp15a receptor in the absence of the plurality of compounds; and if so (c) separately determining the inhibition of activation of the mammalian hp15a receptor for each compound included in the plurality of compounds, so as to thereby identify the compound which inhibits the activation of the mammalian hp15a receptor. In one embodiment, the mammalian hp15a receptor is a human hp15a receptor.

In one embodiment of the above-described methods, the cell is a mammalian cell. In another embodiment, the mammalian cell is non-neuronal in origin. In a further embodiment, the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell or an NIH-3T3 cell.

This invention provides a pharmaceutical composition comprising a compound identified by the above-described methods effective to increase mammalian hp15a receptor activity and a pharmaceutically acceptable carrier.

This invention also provides a pharmaceutical

composition comprising a compound identified by the above-described methods effective to decrease mammalian hp15a receptor activity and a pharmaceutically acceptable carrier.

This invention further provides a method of measuring polypeptide activation in an oocyte expression system such as a Xenopus oocyte expression system or melanophore. In an embodiment, polypeptide activation is determined by measurement of ion channel activity. In another embodiment, polypeptide activation is measured by aequerin luminescence.

Expression of genes in Xenopus oocytes is well known in the art (Coleman, A., 1984; Masu, Y.,et al., 1994) and is performed using microinjection of native mRNA or in vitro synthesized mRNA into frog oocytes. The preparation of in vitro synthesized mRNA can be performed by various standard techniques (Sambrook, et al. 1989) including using T7 polymerase with the mCAP RNA mapping kit (Stratagene).

This invention provides a method of treating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a mammalian hp15a receptor which comprises administering to the subject an amount of a compound which is a mammalian hp15a receptor agonist effective to treat the abnormality. In separate embodiments, the abnormality is a respiratory disorder, asthma, an immune disorder, an endocrine disorder, a neuroendocrine disorder, a cognitive disorder, a memory disorder, a sensory modulation and/or transmission disorder, a motor coordination disorder, a sensory integration disorder, or a dopaminergic function disorder.

This invention provides a method of treating an

abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a mammalian hp15a receptor which comprises administering to the subject an amount of a compound which is a mammalian hp15a receptor antagonist effective to treat the abnormality. In separate embodiments, the abnormality is an endocrine disorder, a neuroendocrine disorder, a sensory modulation and/or transmission disorder, a sensory integration disorder, a dopaminergic function disorder, or a motor coordination disorder.

This invention also provides the use of mammalian hp15a receptors for analgesia.

This invention further provides a process for making a composition of matter which specifically binds to a mammalian hp15a receptor which comprises identifying a chemical compound using any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a mammalian hp15a receptor and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof. In one embodiment, the mammalian hp15a receptor is a human hp15a receptor.

This invention further provides a process for preparing a pharmaceutical composition which comprises admixing a pharmaceutically acceptable amount of a compound identified by any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a mammalian hp15a receptor or a novel structural and functional analog or homolog thereof. In one embodiment, the mammalian hp15a receptor is a human hp15a receptor.

Thus, once the gene for a targeted receptor subtype is cloned, it is placed into a recipient cell which then

expresses the targeted receptor subtype on its surface. This cell, which expresses a single population of the targeted human receptor subtype, is then propagated resulting in the establishment of a cell line. This cell line, which constitutes a drug discovery system, is used in two different types of assays: binding assays and functional assays. In binding assays, the affinity of a compound for both the receptor subtype that is the target of a particular drug discovery program and other receptor subtypes that could be associated with side effects are measured. These measurements enable one to predict the potency of a compound, as well as the degree of selectivity that the compound has for the targeted receptor subtype over other receptor subtypes. The data obtained from binding assays also enable chemists to design compounds toward or away from one or more of the relevant subtypes, as appropriate, for optimal therapeutic efficacy. In functional assays, the nature of the response of the receptor subtype to the compound is determined. Data from the functional assays show whether the comound is acting to inhibit or enhance the activity of the receptor subtype, thus enabling pharmacologists to evaluate compounds rapidly at their ultimate human receptor subtypes targets permitting chemists to rationally design drugs that will be more effective and have fewer or substantially less severe side effects than existing drugs.

Approaches to designing and synthesizing receptor subtype-selective compounds are well known and include traditional medicinal chemistry and the newer technology of combinatorial chemistry, both of which are supported by computer-assisted molecular modeling. With such approaches, chemists and pharmacologists use their knowledge of the structures of the targeted receptor subtype and compounds determined to bind

and/or activate or inhibit activation of the receptor subtype to design and synthesize structures that will have activity at these receptor subtypes.

5   Combinatorial chemistry involves automated synthesis of a variety of novel compounds by assembling them using different combinations of chemical building blocks. The use of combinatorial chemistry greatly accelerates the process of generating compounds. The resulting

10   arrays of compounds are called libraries and are used to screen for compounds (lead comounds) that demonstrate a sufficient level of activity at receptors of interest. Using cominatorial chemistry it is possible to synthesize focused libraries of compounds

15   anticipated to be highly biased toward the receptor target of interest.

Once lead compounds are identified, whether through the use of cominatorial chemistry of traditional medicinal

20   chemistry or otherwise, a variety of homologs and analogs are prepared to facilitate an understanding of the relationship between chemical structure and biological or functional activity. These studies define structure activity relationships which are then

25   used to design drugs with improved potency, selectivity and pharmacokinetic properties. Combinatorial chemistry is also used to rapidly generate a variety of structures for lead optimization. Traditional medicinal chemistry, which involves the synthesis of

30   compounds one at a time, is also used for further refinement and to generate compounds not accessible by automated techniques. Once such drugs are defined the production is scaled up using standard chemical manufacturing methodologies utilized throughout the

35   pharmaceutical and chemistry industry.

This invention will be better understood from the

Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAILS

Materials and methods

Cloning and sequencing of a human receptor (hp15a)

5   A human placenta genomic library in λ dash II ($\approx$1.5 x 10^6 total recombinants; Stratagene, LaJolla, CA) was screened at reduced stringency using overlapping oligonucleotide probes representing transmembrane domains (TMs) III (RW-98/99), V (RW-100/101), and VI

10   (RW-102/103) of the human serotonin 5-HT$_{1D_3}$ receptor ("Clone 11", later identified as 5-HT$_{1D\beta}$). The probes were labeled with [^{32}P]dATP and [^{32}P]dCTP by synthesis with the large fragment of DNA polymerase. Hybridization was performed at reduced stringency

15   conditions: 40°C in a solution containing 25.0% formamide, 5x SSC (1X SSC is 0.15M sodium chloride, 0.015M sodium citrate), 1x Denhardt's solution (0.02% polyvinylpyrrolindone, 0.02% Ficoll, 0.02% bovine serum albumin), and 25 μg/μl sonicated salmon sperm DNA. The

20   filters were washed at 40°C in 0.1x SSC containing 0.1% sodium dodecyl sulfate and exposed at -70°C to Kodak XAR film in the presence of an intensifying screen.

Lambda phage clones hybridizing with the probes were

25   plaque-purified and classified into 14 groups based on the pattern and strength of hybridization with each oligonucleotide. Group 6 consisted of clones that hybridized strongly at reduced stringency with TM3 oligos. One clone in that group, hp15a, was

30   prioritized for analysis after positive hybridization with oligonucleotides representing the TM VI domain of the novel GPCR sequence designated G21 (later identified as 5-HT$_{1A}$; oligos RW-96/97). Phage DNA from each of these clones was amplified by liquid lysis and

35   isolated according to standard methods for Southern blot analysis (Southern, 1975; Sambrook et al., 1989). DNA was digested with PstI, BglII, or both enzymes,

separated by agarose gel electrophoresis, and blotted to nitrocellulose membranes for hybridization at reduced stringency with the TM VI (G21) and TM III (Clone 11) oligos described above, designated RW-96/97 and RW-98/99, respectively.

The oligo sequences are:

RW-96:

5'-GGCATCATCATGGGCACCTTCATCCTCTGCTGGCTGCCCTTCTTC-3'

(Seq. I.D. No. 3)

RW-97:

5'-GCAGAAGGGCAGAACAAGAGCCACGATGAAGAAGGGCAGCCAGCA-3'

(Seq. I.D. No. 4)

RW-98:

5'-TGGCTGTCATCGGACATCACTTGTTGCACTGCCTCCATCCTGCAC-3'

(Seq. I.D. No. 5)

RW-99:

5'-GTAGCGGTCCAGGGCGATGACACAGAGGTGCAGGATGGAGGCAGT-3'

(Seq. I.D. No. 6)

RW-100:

5'-ATCCTCTACACTGTCTACTCCACGGTGGGTGCTTTCTACTTCCCC-3'

(Seq. I.D. No. 7)

RW-101:

5'-GCCATAGAGGGCGATGAGGAGCAGGGTGGGGAAGTAGAAAGCACC-3'

(Seq. I.D. No. 8)

RW-102:

5'-CTAGGGATCATTTTGGGAGCCTTTATTGTGTGTTGGCTACCCTTCT-3'

(Seq. I.D. No. 9)

RW-103:

5'-GATAGGCATCACTAGGGAGATGATGAAGAAGGGTAGCCAACACACA-3'
(Seq. I.D. No. 10)

A 223bp PstI fragment of the hp15a gene hybridizing with the TM III oligos was subcloned for further analysis into pUC18 (Pharmacia, Piscataway, NJ) and designated K28. Sequence analysis revealed that the fragment encoded a novel GPCR-like TM III domain with an unusual predicted amino acid sequence motif, "LGRY", rather than the commonly observed "LDRY" sequence at that location. In an attempt to obtain the entire coding region of the putative GPCR a ~3 kb BglII fragment that hybridized with the same probes was subcloned into pUC18 (designated K49). Sequence analysis showed that the fragment could encode TMs I through VII but not a starting methionine, indicating that the N-terminus was truncated. To obtain the full 5' coding region a ~750 bp BamHI/HindIII fragment of the genomic clone hp15a was subcloned and sequenced. Since this fragment contained an in-frame start codon and stop codons further upstream in all three reading frames, it appeared to encode the native N-terminus of the novel receptor. The BamHI/HindIII fragment was ligated with a HindIII/EcoRI fragment of the previously described BglII fragment into pUC18 for subsequent isolation of a BamHI/EcoRI fragment encoding the complete coding region. This fragment was blunted and ligated into the expression vector pcEXV-3 (Miller and Germain, 1986); a single colony containing the full-length hp15a DNA in the correct orientation (designated K90) was selected for amplification, sequencing, and expression studies. Nucleotide sequence analysis was accomplished by the Sanger dideoxy nucleotide chain termination method (Sanger et al., 1977) on denatured double-stranded plasmid templates, using Sequenase (US Biochemical Corp., Cleveland, OH).

Cell culture

COS-7 cells are grown on 150 mm plates in DMEM with supplements (Dulbecco's Modified Eagle Medium with 10% bovine calf serum, 4 mM glutamine, 100 units/ml penicillin/100 μg/ml streptomycin) at 37°C, 5% CO_2. Stock plates of COS-7 cells are trypsinized and split 1:6 every 3-4 days.

Human embryonic kidney 293 cells are grown on 150 mm plates in DMEM with supplements (10% bovine calf serum, 4 mM glutamine, 100 units/ml penicillin/100 μg/ml streptomycin) at 37°C, 5% CO_2. Stock plates of 293 cells are trypsinized and split 1:6 every 3-4 days.

Mouse fibroblast LM(tk-) cells are grown on 150 mm plates in D-MEM with supplements (Dulbecco's Modified Eagle Medium with 10% bovine calf serum, 4 mM glutamine, 100 units/ml penicillin/100 μg/ml streptomycin) at 37°C, 5% CO_2. Stock plates of LM(tk-) cells are trypsinized and split 1:10 every 3-4 days.

Chinese hamster ovary (CHO) cells were grown on 150 mm plates in HAM's F-12 medium with supplements (10% bovine calf serum, 4 mM L-glutamine and 100 units/ml penicillin/ 100 ug/ml streptomycin) at 37°C, 5% CO_2. Stock plates of CHO cells are trypsinized and split 1:8 every 3-4 days.

Mouse embryonic fibroblast NIH-3T3 cells are grown on 150 mm plates in Dulbecco's Modified Eagle Medium (DMEM) with supplements (10% bovine calf serum, 4 mM glutamine, 100 units/ml penicillin/100 μg/ml streptomycin) at 37°C, 5% CO2. Stock plates of NIH-3T3 cells are trypsinized and split 1:15 every 3-4 days.

Sf9 and Sf21 cells are grown in monolayers on 150 mm tissue culture dishes in TMN-FH media supplemented with

10% fetal calf serum, at 27°C, no CO_2. High Five insect cells are grown on 150 mm tissue culture dishes in Ex-Cell 400™ medium supplemented with L-Glutamine, also at 27°C, no CO_2.

Transient transfection

Receptors studied may be transiently transfected into COS-7 cells by the DEAE-dextran method using 1 μg of DNA /10^6 cells (Cullen, 1987). In addition, Schneider 2 Drosophila cells may be cotransfected with vectors containing the receptor gene under control of a promoter which is active in insect cells, and a selectable resistance gene, eg., the G418 resistant neomycin gene, for expression of the polypeptides disclosed herein.

Stable transfection

DNA encoding the human receptor disclosed herein may be co-transfected with a G-418 resistant gene into the human embryonic kidney 293 cell line by a calcium phosphate transfection method (Cullen, 1987). Stably transfected cells are selected with G-418.

Membrane preparations

After transfection, Cos-7 cells are grown for 48 h in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. Cells are harvested by scraping into Dulbecco's phosphate buffered saline (PBS), and recovered by centrifugation at 200 X g for 1 min at 4°C. Cells are lysed by suspension in ice-cold homogenizing buffer (20mM Tris-HC1, 5mM EDTA, pH 7.4) followed by sonication for 7 sec. Cell lysates are centrifuged at 200 X g for 5 min at 4°C. Supernatants were centrifuged at 40,000 X g for 20 min at 4°C, and the membrane protein pellets are washed once with homogenizing buffer.

LM(tk-) cells stably transfected with the DNA encoding
the human receptor disclosed herein may be routinely
converted from an adherent monolayer to a viable
suspension. Adherent cells are harvested with trypsin
at the point of confluence, resuspended in a minimal
volume of complete DMEM for a cell count, and further
diluted to a concentration of 10^6 cells/ml in suspension
media (10% bovine calf serum, 10% 10X Medium 199
(Gibco), 9 mM $NaHCO_3$, 25 mM glucose, 2 mM L-glutamine,
100 units/ml penicillin/100 μg/ml streptomycin, and
0.05% methyl cellulose). Cell suspensions are
maintained in a shaking incubator at 37°C, 5% CO_2 for
24 hours. Membranes harvested from cells grown in this
manner may be stored as large, uniform batches in
liquid nitrogen. Alternatively, cells may be returned
to adherent cell culture in complete DMEM by
distribution into 96-well microtiter plates coated with
poly-D-lysine (0.01 mg/ml) followed by incubation at
37°C, 5% CO_2 for 24 hours.

Generation of baculovirus

The coding region of DNA encoding the human receptor
disclosed herein may be subcloned into pBlueBacIII into
existing restriction sites or sites engineered into
sequences 5' and 3' to the coding region of the
polypeptides. To generate baculovirus, 0.5 μg of viral
DNA (BaculoGold) and 3 μg of DNA construct encoding a
polypeptide may be co-transfected into 2 x 10^6
Spodoptera frugiperda insect Sf9 cells by the calcium
phosphate co-precipitation method, as outlined in by
Pharmingen (in "Baculovirus Expression Vector System:
Procedures and Methods Manual"). The cells then are
incubated for 5 days at 27°C.

The supernatant of the co-transfection plate may be
collected by centrifugation and the recombinant virus
plaque purified. The procedure to infect cells with

virus, to prepare stocks of virus and to titer the virus stocks are as described in Pharmingen's manual.

Radioligand binding assays

Cells may be screened for the presence of endogenous human receptor using radioligand binding or functional assays (described in detail in the following experimental description). Cells with either no or a low level of the endogenous human receptor disclosed herein present may be transfected with the human receptor.

Transfected cells from culture flasks are scraped into 5 ml of Tris-HCl, 5mM EDTA, pH 7.5, and lysed by sonication. The cell lysates are centrifuged at 1000 rpm for 5 min. at 4°C, and the supernatant is centrifuged at 30,000 x g for 20 min. at 4°C. The pellet is suspended in binding buffer (50 mM Tris-HCl, 5 mM $MgSO_4$, 1 mM EDTA at pH 7.5 supplemented with 0.1% BSA, 2 μg/ml aprotinin, 0.5 mg/ml leupeptin, and 10 μg/ml phosphoramidon). Optimal membrane suspension dilutions, defined as the protein concentration required to bind less than 10% of the added radioligand, are added to 96-well polpropylene microtiter plates containing radiolabeled compound, unlabeled compounds, and binding buffer to a final volume of 250 μl. In equilibrium saturation binding assays membrane preparations are incubated in the presence of increasing concentrations of radiolabeled compound. The binding affinities of the different compounds are determined in equilibrium competition binding assays, using radiolabeled compound in the presence of ten to twelve different concentrations of the displacing ligands. Binding reaction mixtures are incubated for 1 hr at 30°C, and the reaction stopped by filtration through GF/B filters treated with 0.5% polyethyleneimine, using a cell harvester.

Radioactivity may be measured by scintillation counting and data are analyzed by a computerized non-linear regression program. Non-specific binding is defined as the amount of radioactivity remaining after incubation of membrane protein in the presence of unlabeled ligand. Protein concentration may be measured by the Bradford method using Bio-Rad Reagent, with bovine serum albumin as a standard.

Functional assays

Cells may be screened for the presence of endogenous mammalian receptor using radioligand binding or functional assays (described in detail in the above or following experimental description, respectively). Cells with no or a low level of endogenous receptor present may be transfected with the mammalian receptor for use in the following functional assays.

A wide spectrum of assays can be employed to screen for the presence of orphan receptor ligands. These assays range from traditional measurements of phosphatidyl inositol, cAMP, Ca^{++}, and K^+, for example; to systems measuring these same second messengers but which have been modified or adapted to be higher throughput, more generic and more sensitive; to cell based platforms reporting more general cellular events resulting from receptor activation such as metabolic changes, differentiation, cell division/proliferation, for example; to high level organism assays which monitor complex physiological or behavioral changes thought to be involved with receptor activation including cardiovascular, analgesic, orexigenic, anxiolytic, and sedation effects, for example.

Cyclic AMP (cAMP) formation assay

The receptor-mediated stimulation or inhibition of cyclic AMP (cAMP) formation may be assayed in

transfected cells expressing the mammalian receptors. Cells are plated in 96-well plates and incubated in Dulbecco's phosphate buffered saline (PBS) supplemented with 10 mM HEPES, 5mM theophylline, 2 μg/ml aprotinin, 0.5 mg/ml leupeptin, and 10 μg/ml phosphoramidon for 20 min at 37°C, in 5% CO_2. Test compounds are added with or without 10μM forskolin and incubated for an additional 10 min at 37°C. The medium is then aspirated and the reaction stopped by the addition of 100 mM HCl. The plates are stored at 4°C for 15 min, and the cAMP content in the stopping solution measured by radioimmunoassay. Radioactivity may be quantified using a gamma counter equipped with data reduction software.

Arachidonic acid release assay

Cells stably transfected with the mammalian receptor are seeded into 96 well plates and grown for 3 days in HAM's F-12 with supplements. ^{3}H-arachidonic acid (specific activity = 0.75 μCi/ml) is delivered as a 100 μL aliquot to each well and samples were incubated at 37° C, 5% CO_2 for 18 hours. The labeled cells are washed three times with 200 μL HAM's F-12. The wells are then filled with medium (200 μL) and the assay is initiated with the addition of peptides or buffer (22 μL). Cells are incubated for 30 min at 37°C, 5% CO_2. Supernatants are transferred to a microtiter plate and evaporated to dryness at 75°C in a vacuum oven. Samples are then dissolved and resuspended in 25 μL distilled water. Scintillant (300 μL) is added to each well and samples are counted for ^{3}H in a Trilux plate reader. Data are analyzed using nonlinear regression and statistical techniques available in the GraphPAD Prism package (San Diego, CA).

Intracellular calcium mobilization assay

The intracellular free calcium concentration may be

measured by microspectroflourometry using the fluorescent indicator dye Fura-2/AM (Bush et al, 1991). Stably transfected cells are seeded onto a 35 mm culture dish containing a glass coverslip insert. Cells are washed with HBS and loaded with 100 μL of Fura-2/AM (10 μM) for 20 to 40 min. After washing with HBS to remove the Fura-2/AM solution, cells are equilibrated in HBS for 10 to 20 min. Cells are then visualized under the 40X objective of a Leitz Fluovert FS microscope and fluorescence emission is determined at 510 nM with excitation wavelengths alternating between 340 nM and 380 nM. Raw fluorescence data are converted to calcium concentrations using standard calcium concentration curves and software analysis techniques.

Phosphoinositide metabolism assay

Cells stably expressing the mammalian receptor cDNA are plated in 96-well plates and grown to confluence. The day before the assay the growth medium is changed to 100 μl of medium containing 1% serum and 0.5 μCi [^{3}H]myo-inositol, and the plates are incubated overnight in a CO_2 incubator (5% CO_2 at 37^{0}C). Immediately before the assay, the medium is removed and replaced by 200 μL of PBS containing 10 mM LiCl, and the cells are equilibrated with the new medium for 20 min. During this interval cells are also equilibrated with the antagonist, added as a 10 μL aliquot of a 20-fold concentrated solution in PBS. The ·[^{3}H]inositol-phosphates accumulation from inositol phospholipid metabolism may be started by adding 10 μL of a solution containing the agonist. To the first well 10 μL may be added to measure basal accumulation, and 11 different concentrations of agonist are assayed in the following 11 wells of each plate row. All assays are performed in duplicate by repeating the same additions in two consecutive plate rows. The plates are incubated in a

CO_2 incubator for 1 hr. The reaction may be terminated by adding 15 μL of 50% v/v trichloroacetic acid (TCA), followed by a 40 min. incubation at 4 ^{0}C. After neutralizing TCA with 40 μL of 1 M Tris, the content of the wells may be transferred to a Multiscreen HV filter plate (Millipore) containing Dowex AG1-X8 (200-400 mesh, formate form). The filter plates are prepared adding 200 μL of Dowex AG1-X8 suspension (50% v/v, water: resin) to each well. The filter plates are placed on a vacuum manifold to wash or elute the resin bed. Each well is washed 2 times with 200 μL of water, followed by 2 x 200 μL of 5 mM sodium tetraborate/60 mM ammonium formate. The [^{3}H]IPs are eluted into empty 96-well plates with 200 μL of 1.2 M ammonium formate/0.1 formic acid. The content of the wells is added to 3 ml of scintillation cocktail, and the radioactivity is determined by liquid scintillation counting.

GTPγS functional assay

Membranes from cells transfected with the mammalian receptors are suspended in assay buffer (50 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4) supplemented with 0.1% BSA, 0.1% bacitracin and 10 μM GDP. Membranes are incubated on ice for 20 minutes, transferred to a 96-well Millipore microtiter GF/C filter plate and mixed with GTPγ^{35}S (e.g., 250,000 cpm/sample, specific activity ~1000 Ci/mmol) plus or minus GTPγS (final concentration = 100 μM). Final membrane protein concentration $\approx$ 90 μg/ml. Samples are incubated in the presence or absence of porcine galanin (final concentration = 1 μM) for 30 min. at room temperature, then filtered on a Millipore vacuum manifold and washed three times with cold assay buffer. Samples collected in the filter plate are treated with scintillant and counted for ^{35}S in a Trilux (Wallac) liquid scintillation counter. It is expected that optimal results are obtained when the mammalian receptor

membrane preparation is derived from an appropriately engineered heterologous expression system, i.e., an expression system resulting in high levels of expression of the mammalian receptor and/or expressing G-proteins having high turnover rates (for the exchange of GDP for GTP). GTPγS assays are well-known in the art, and it is expected that variations on the method described above, such as are described by e.g., Tian et al. (1994) or Lazareno and Birdsall (1993), may be used by one of ordinary skill in the art.

MAP kinase assay

MAP kinase (mitogen activated kinase) may be monitored to evaluate receptor activation. MAP kinase is activated by multiple pathways in the cell. A primary mode of activation involves the ras/raf/MEK/MAP kinase pathway. Growth factor (tyrosine kinase) receptors feed into this pathway via SHC/Grb-2/SOS/ras. Gi coupled receptors are also known to activate ras and subsequently produce an activation of MAP kinase. Receptors that activate phospholipase C (Gq and G11) produce diacylglycerol (DAG) as a consequence of phosphatidyl inositol hydrolysis. DAG activates protein kinase C which in turn phosphorylates MAP kinase.

MAP kinase activation can be detected by several approaches. One approach is based on an evaluation of the phosphorylation state, either unphosphorylated (inactive) or phosphorylated (active). The phosphorylated protein has a slower mobility in SDS-PAGE and can therefore be compared with the unstimulated protein using Western blotting. Alternatively, antibodies specific for the phosphorylated protein are available (New England Biolabs) which can be used to detect an increase in the phosphorylated kinase. In either method, cells are

stimulated with the mitogen and then extracted with Laemmli buffer. The soluble fraction is applied to an SDS-PAGE gel and proteins are transferred electrophoretically to nitrocellulose or Immobilon.

5   Immunoreactive bands are detected by standard Western blotting technique. Visible or chemiluminescent signals are recorded on film and may be quantified by densitometry.

10   Another approach is based on evaluation of the MAP kinase activity via a phosphorylation assay. Cells are stimulated with the mitogen and a soluble extract is prepared. The extract is incubated at 30°C for 10 min with gamma-32-ATP, an ATP regenerating system, and a

15   specific substrate for MAP kinase such as phosphorylated heat and acid stable protein regulated by insulin, or PHAS-I. The reaction is terminated by the addition of H_3PO_4 and samples are transferred to ice. An aliquot is spotted onto Whatman P81

20   chromatography paper, which retains the phosphorylated protein. The chromatrography paper is washed and counted for ^{32}P in a liquid scintillation counter. Alternatively, the cell extract is incubated with gamma-32-ATP, an ATP regenerating system, and

25   biotinylated myelin basic protein bound by streptavidin to a filter support. The myelin basic protein is a substrate for activated MAP kinase. The phosphorylation reaction is carried out for 10 min at 30°C. The extract can then by aspirated through the filter, which

30   retains the phosphorylated myelin basic protein. The filter is washed and counted for ^{32}P by liquid scintillation counting.

Cell proliferation assay

35   Receptor activation of a G protein-coupled receptor may lead to a mitogenic or proliferative response which can be monitored via ^{3}H-thymidine uptake. When cultured

cells are incubated with ^{3}H-thymidine, the thymidine translocates into the nuclei where it is phosphorylated to thymidine triphosphate. The nucleotide triphosphate is then incorporated into the cellular DNA at a rate that is proportional to the rate of cell growth. Typically, cells are grown in culture for 1-3 days. Cells are forced into quiescence by the removal of serum for 24 hrs. A mitogenic agent is then added to the media. 24 hrs later, the cells are incubated with ^{3}H-thymidine at specific activities ranging from 1 to 10 uCi/ml for 2-6 hrs. Harvesting procedures may involve trypsinization and trapping of cells by filtration over GF/C filters with or without a prior incubation in TCA to extract soluble thymidine. The filters are processed with scintillant and counted for ^{3}H by liquid scintillation counting. Alternatively, adherant cells are fixed in MeOH or TCA, washed in water, and solubilized in 0.05% deoxycholate/0.1 N NaOH. The soluble extract is transferred to scintillation vials and counted for ^{3}H by liquid scintillation counting.

Promiscuous second messenger assays

It is not possible to predict, _a priori_ and based solely upon the GPCR sequence, which of the cell's many different signaling pathways any given orphan receptor will naturally use. It is possible, however, to coax receptors of different functional classes to signal through a pre-selected pathway through the use of promiscuous G_α subunits. For example, by providing a cell based receptor assay system with an endogenously supplied promiscuous G_α subunit such as $G_{\alpha 16}$ or a chimeric G_α subunit such as $G_{\alpha q z}$, a GPCR, which might normally prefer to couple through a specific signaling pathway (e.g., G_s, G_i, G_q, G_o, etc.), can be made to couple through the pathway defined by the promiscuous G_α subunit and upon agonist activation produce the second messenger associated with that subunit's pathway.

In the case of $G_{\alpha 16}$ and/or $G_{\alpha qz}$ this would involve activation of the G_q pathway and production of the second messenger phosphotidyl inositol. Through the use of similar strategies and tools, it is possible to bias receptor signaling through pathways producing other second messengers such as Ca^{++}, cAMP, and ^+K currents, for example.

It follows that the promiscuous interaction of the exogenously supplied G_α subunit with the orphan receptor alleviates the need to carry out a different assay for each possible signaling pathway and increases the chances of detecting a functional signal upon receptor activation.

Microphysiometric measurement of orphan receptor mediated extracellular acidification rates

Because cellular metabolism is intricately involved in a broad range of cellular events (including receptor activation of multiple messenger pathways), the use of microphysiometric measurements of cell metabolism can in principle provide a generic assay of cellular activity arising from the activation of any orphan receptor regardless of the specifics of the receptor's signaling pathway.

General guidelines for transient receptor expression, cell preparation and microphysiometric recording are described elsewhere (Salon, J.A. and Owicki, J.A., 1996). Orphan receptors and/or control vectors are transiently expressed in CHO-K1 cells, by liposome mediated transfection according to the manufacturers recommendations (LipofectAMINE, GibcoBRL, Bethesda, MD), and maintained in Ham's F-12 complete (10% serum). 24 hours post transfection, the cells are harvested and 3×10^5 cells seeded into microphysiometet capsules. Cells are allowed to attach to the capsule membrane for

an additional 24 hours; during the last 16 hours, the cells are switched to serum-free F-12 complete to minimize ill-defined metabolic stimulation caused by assorted serum factors. On the day of the experiment the cell capsules are transferred to the microphysiometer and allowed to equilibrate in recording media (low buffer RPMI 1640, no bicarbonate, no serum (Molecular Devices Corporation, Sunnyvale, CA) containing 0.1% fatty acid free BSA), during which a baseline measurement of basal metabolic activity is established.

A standard recording protocol specifies a 100μl/min flow rate, with a 2 min total pump cycle which includes a 30 sec flow interruption during which the acidification rate measurement is taken. Ligand challenges involve a 1 min 20 sec exposure to the sample just prior to the first post challenge rate measurement being taken, followed by two additional pump cycles for a total of 5 min 20 sec sample exposure. Typically, drugs in a primary screen are presented to the cells at 10μM final concentration. Ligand samples are then washed out and the acidification rates reported are expressed as a percentage increase of the peak response over the baseline rate observed just prior to challenge.

Identification of orphan receptor ligands

Clearly, an important aspect of understanding orphan receptors is the identification and characterization of their ligands. The scope and structural diversity of activating ligands (agonists) anticipated to be discovered for orphans is represented by the known universe of ligands for the GPCR superfamily. These range from large viral coat proteins and glycoproteins, to peptides, lipids, small molecules, and even activating ions. The diversity can be further expanded

upon if we consider the many known synthetic antagonists specific for GPCR subtypes.

Discrete GPCR ligand library

Functional assays of orphan receptors include a preliminary test of a small library of compounds containing representative agonists for all known GPCRs as well as other compounds which may be agonists for prospective GPCRs or which may be effectors for targets peripherally involved with GPCRs. The collection currently comprises approximately 180 compounds, (including small molecules, hormones, preprohormones, and peptides, for example), for more than 45 described classes of GPCRs (serotonin, dopamine, noradrenalin, opiods, etc.) and additionally includes ligands for known or suspected but not necessarily pharmacologically characterized or cloned GPCR families. The diversity of the library can be expanded to include agonist and antagonist compounds specific for GPCR subtypes, combinatorial peptide and/or small molecule libraries, natural product collections, and the like. To facilitate robotic handling, the substances are distributed as either separate or pooled compound concentrates in 96 well plates and stored frozen as ready to use reagent plates.

Peptide transmitter cDNA library

It is anticipated that a large portion of orphan receptors will have peptide or protein molecules as their natural ligands. Accordingly, approaches employing the expression cloning of novel peptide transmitters using assay systems and cDNA libraries tailored to this task are a viable approach to the problem of identifying orphan receptor ligands.

Isolation of endogenous ligands

Due to the limited understanding of the structural

basis of transmitter diversity, it is very likely that successful identification of orphan receptor ligands will come not through efforts that rely solely on screening synthetic chemical or peptide libraries, but rather through the screening of ligand rich biological extracts from organisms and tissues that express the receptor itself as well. The logic of this hypothesis is that where nature has evolved a regulatory system based on a novel receptor it must also provide the means to activate the receptor via a novel endogenous transmitter substance. Accordingly, it is important in outlining a strategy to include the orphan receptor based screening of extracts derived from naturally occurring biological sources and the subsequent purification and characterization of any orphan receptor linked biological activity present in said extracts.

A general approach is to screen high resolution HPLC fractions of various tissue extracts for orphan receptor activity, employing one or more cellular based assays as described elsewhere. In general, a receptor based assay system employing reporter cells, which either transiently or stably express a particular orphan receptor(s), will be challenged with HPLC fractions derived from tissues thought to harbor transmitter substances and monitor signal transduction readouts for heterotrimeric G protein activation. To circumvent the problem of endogenous GPCRs (orphan or extaneous) in the reporter lines that may be activated by one or more endogenous transmitters in the extracts, the parent host cell lines (i.e. not heterologously expressing the orphan receptor) will be tested in parallel. Positive hits for orphan receptor linked activity will be evidenced by signaling present in the cell line heterologously expressing the orphan receptor but absent in the parent line. Tissue sources

for extraction will be chosen by several criteria, including the localization of the orphan receptor itself, the relative abundance of known transmitter substances, and the potential involvement of the tissue in important disease states. Extraction procedures will depend upon the structural class of ligand being sought after and could include but not be restricted to; neutral aqueous extraction for protein molecules, acid extraction for peptide molecules and small molecule chemical transmitters, and organic solvent extraction for lipid or sterol molecules.

Purification of orphan receptor linked biological activity will depend upon the structural characteristic of the transmitter substance, but could include various low, medium and high pressure chromatographic methods based on size exclusion, anion/cation, hydrophobic, and affinity interaction matrices and could employ either normal or reversed phase conditions. Preparative electrophoresis in one and two dimensions would also, in some circumstances, be a viable approach for purification.

In addition to various signal transduction assays which would be used to track bio-activity during purification, various biophysical methods would be employed to analyze the complexity and structural characteristics of the purified fractions. These methods would include, but not be limited to, UV-vis absorbance spectroscopy, proteolytic fragmentation, mass spectrometry, amino acid sequencing, and ultimately nuclear magnetic resonance spectrometry and/or X-ray crystallographic determination of the purified transmitter molecule's 3-dimensional structure.

Receptor/G protein co-transfection studies
A strategy for determining whether the hp15a receptor

can couple preferentially to selected G proteins involves co-transfection of hp15a receptor cDNA into a host cell together with the cDNA for a G protein alpha sub-unit. Examples of G alpha sub-units include members of the Gαi/Gαo class (including Gαt2 and Gαz), the Gαq class, the Gαs class, and the Gα12/13 class. A typical procedure involves transient transfection into a host cell such as COS-7. Other host cells may be used. A key consideration is whether the cell has a downstream effector (a particular adenylate cyclase, phospholipase C, or channel isoform, for example) to support a functional response through the G protein under investigation. G protein beta gamma sub-units native to the cell are presumed to complete the G protein heterotrimer; otherwise specific beta and gamma sub-units may be co-transfected as well. Additionally, any individual or combination of alpha, beta, or gamma subunits may be co-transfected to optimize the functional signal mediated by the receptor.

The receptor/G alpha co-transfected cells are evaluated in a binding assay, in which case the radioligand binding may be enhanced by the presence of the optimal G protein coupling or in a functional assay designed to test the receptor/G protein hypothesis. In one example, the hp15a receptor may be hypothesized to inhibit cAMP accumulation through coupling with G alpha sub-units of the Gαi/Gαo class. Host cells co-transfected with hp15a receptor cDNA and appropriate G alpha sub-unit cDNA are stimulated with forskolin +/- hp15a receptor agonist, as described above in cAMP methods. Intracellular cAMP is extracted for analysis by radioimmunoassay. Other assays may be substituted for cAMP inhibition, including GTPγ^{35}S binding assays and inositol phosphate hydrolysis assays. Host cells transfected with hp15a receptor cDNA minus G alpha or with G alpha minus hp15a receptor cDNA would be tested

simultaneously as negative controls. hp15a receptor expression in transfected cells may be confirmed in ^{125}I-hp15a protein binding studies using membranes from transfected cells. G alpha expression in transfected cells may be confirmed by Western blot analysis of membranes from transfected cells, using antibodies specific for the G protein of interest.

The efficiency of the transient transfection procedure is a critical factor for signal to noise in an inhibitory assay, much more so than in a stimulatory assay. If a positive signal present in all cells (such as forskolin-stimulated cAMP accumulation) is inhibited only in the fraction of cells successfully transfected with receptor and G alpha, the signal to noise ratio will be poor. One method for improving the signal to noise ratio is to create a stably transfected cell line in which 100% of the cells express both the receptor and the G alpha subunit. Another method involves transient co-transfection with a third cDNA for a G protein-coupled receptor which positively regulates the signal which is to be inhibited. If the co-transfected cells simultaneously express the stimulatory receptor, the inhibitory receptor, and a requisite G protein for the inhibitory receptor, then a positive signal may be elevated selectively in transfected cells using a receptor-specific agonist. An example involves co-transfection of COS-7 cells with 5-HT4 cDNA, hp15a receptor cDNA, and a G alpha sub-unit cDNA. Transfected cells are stimulated with a 5-HT4 agonist +/- hp15a protein. Cyclic AMP is expected to be elevated only in the cells also expressing the hp15a receptor and the G alpha subunit of interest, and a hp15a receptor-dependent inhibition may be measured with an improved signal to noise ratio.

It is to be understood that the cell lines described

herein are merely illustrative of the methods used to evaluate the binding and function of the mammalian receptors of the present invention, and that other suitable cells may be used in the assays described
5   herein.

Methods for recording currents in Xenopus oocytes

Female *Xenopus laevis* (Xenopus-1, Ann Arbor, MI) are anesthetized in 0.2% tricain (3-aminobenzoic acid ethyl
10   ester, Sigma Chemical Corp.) and a portion of ovary is removed using aseptic technique (Quick and Lester, 1994). Oocytes are defolliculated using 2 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ) in a solution containing 87.5 mM NaCl, 2 mM KCl, 2
15   mM $MgCl_2$ and 5 mM HEPES, pH 7.5. Oocytes may be injected (Nanoject, Drummond Scientific, Broomall, PA) with mammalian mRNA. Other oocytes may be injected with a mixture of mammalian mRNA and mRNA encoding the genes for G-protein-activated inward rectifiers (GIRK1 and
20   GIRK4, U.S. Patent Nos. 5,734,021 and 5,728,535). Genes encoding G-protein inwardly rectifying K^+ (GIRK) channels 1 and 4 (GIRK1 and GIRK4) were obtained by PCR using the published sequences (Kubo et al., 1993; Dascal et al., 1993; Krapivinsky et al., 1995 and
25   1995b) to derive appropriate 5' and 3' primers. Human heart cDNA was used as template together with the primers
5'-CGCGGATCCATTATGTCTGCACTCCGAAGGAAATTTG-3' (Seq. I.D. No. 15) and
30   5'-CGCGAATTCTTATGTGAAGCGATCAGAGTTCATTTTTC -3' (Seq. I.D. No. 16) for GIRK1 and
5'-GCGGGATCCGCTATGGCTGGTGATTCTAGGAATG-3' (Seq. I.D. No. 17) and
5'- CCGGAATTCCCCTCACACCGAGCCCCTGG-3' (Seq. I.D. No. 18)
35   for GIRK4.
In each primer pair, the upstream primer contained a BamHI site and the downstream primer contained an EcoRI

site to facilitate cloning of the PCR product into pcDNA1-Amp (Invitrogen). The transcription template for the mammalian receptor may be similarly obtained. mRNAs are prepared from separate DNA plasmids containing the complete coding regions of the mammalian receptor, GIRK1, and GIRK4. Plasmids are linearized and transcribed using the T7 polymerase ("Message Machine", Ambion). Alternatively, mRNA may be translated from a template generated by PCR, incorporating a T7 promoter and a poly A^+ tail. Each oocyte receives 2 ng each of GIRK1 and GIRK4 mRNA in combination with 25 ng of mammalian receptor mRNA. After injection of mRNA, oocytes are incubated at 16° C on a rotating platform for 3-8 days. Dual electrode voltage clamp ("GeneClamp", Axon Instruments Inc., Foster City, CA) is performed using 3 M KCl-filled glass microelectrodes having resistances of 1-3 Mohms. Unless otherwise specified, oocytes are voltage clamped at a holding potential of -80 mV. During recordings, oocytes are bathed in continuously flowing (2-5 ml/min) medium containing 96 mM NaCl, 2 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM HEPES, pH 7.5 ("ND96"), or, in the case of oocytes expressing GIRK1 and GIRK4, elevated K^+ containing 96 mM KCl, 2 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM HEPES, pH 7.5 ("hK"). Drugs are applied by switching from a series of gravity fed perfusion lines.

Heterologous expression of GPCRs in *Xenopus* oocytes has been widely used to determine the identity of signaling pathways activated by agonist stimulation (Gundersen et al., 1983; Takahashi et al., 1987). Activation of the phospholipase C (PLC) pathway is assayed by applying test compound in ND96 solution to oocytes previously injected with mRNA for the mammalian receptor and observing inward currents at a holding potential of -80 mV. The appearance of currents that reverse at -25 mV and display other properties of the Ca^{++}-activated Cl^-

(chloride) channel is indicative of mammalian receptor-activation of PLC and release of IP3 and intracellular Ca^{++}. Such activity is exhibited by GPCRs that couple to G_q.

Measurement of inwardly rectifying K^+ (potassium) channel (GIRK) activity is monitored in oocytes that have been co-injected with mRNAs encoding the mammalian receptor, GIRK1, and GIRK4. The two GIRK gene products co-assemble to form a G-protein activated potassium channel known to be activated (i.e., stimulated) by a number of GPCRs that couple to G_i or G_o (Kubo et al., 1993; Dascal et al., 1993). Oocytes expressing the mammalian receptor plus the two GIRK subunits are tested for test compound responsivity by measuring K^+ currents in elevated K^+ solution (hK). Activation of inwardly rectifying currents that are sensitive to 300 μM Ba^{++} signifies the mammalian receptor coupling to a G_i or G_o pathway in the oocytes.

Localization Studies

Development of probes: Using full length cDNA encoding the hp-15a receptor as a template, polymerase chain reaction (PCR) was used to amplify a 365 base pair fragment corresponding to nucleotides 181-546 of the coding sequence. PCR generated fragments were subcloned into a plasmid vector pGEM 7zf, which contains sp6 and T7 RNA polymerase promoter sites. This construct was linearized with BAM HI and T7 RNA polymerase was used to synthesize radiolabeled antisense strands of RNA.

A probe coding for rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene, a constitutively expressed protein, was used concurrently. GAPDH is expressed at a relatively constant level in most tissue and its detection is used to compare expression levels of the

hp-15a gene in different tissue.

Synthesis of probes: hp-15a and GAPDH cDNA sequences preceded by phage polymerase promoter sequences were used to synthesize radiolabeled riboprobes. Conditions for the synthesis of riboprobes were: 0.25-1.0 μg linearized template, 1.5 μl of ATP, GTP, UTP (10 mM each), 3 μl dithiothreitol (0.1M), 30 units RNAsin RNAse inhibitor, 0.5-1.0 μl (15-20 units/μl) RNA polymerase, 7.0 μl transcription buffer (Promega Corp.), and 12.5 μl α^{32}P-CTP (specific activity 3,000Ci/mmol). 0.1 mM CTP (0.02-1.0 μl) was added to the reactions, and the volumes were adjusted to 35 μl with DEPC-treated water. Labeling reactions were incubated at 37°C for 60 minutes, after which 3 units of RQ1 RNAse-free DNAse (Promega Corp.) were added to digest the template. Riboprobes were separated from unincorporated nucleotides using Microspin S-300 columns (Pharmacia Biotech). TCA precipitation and liquid scintillation spectrometry were used to measure the amount of label incorporated into the probe. A fraction of all riboprobes synthesized were size-fractionated on 0.25 mm thick 7M urea, 4.5% acrylamide sequencing gels. These gels were apposed to screens and the autoradiograph scanned using a phosphorimager (Molecular Dynamics) to confirm that the probes synthesized were full-length and not degraded.

Solution hybridization/ribonuclease protection assay: For solution hybridization 2.0 μg of mRNA isolated from tissues were used. Negative controls consisted of 30 μg transfer RNA (tRNA) or no tissue blanks. All mRNA samples were placed in 1.5 ml microfuge tubes and vacuum dried. Hybridization buffer (40 μl of 400 mM NaCl, 20 mM Tris, pH 6.4, 2 mM EDTA, in 80% formamide) containing 0.25-2.0 E^6 counts of each probe were added to each tube. Samples were heated at 90°C for 5 min,

after which the temperature was lowered to 45 or 55°C for hybridization.

After hybridization for 14-18 hr, the RNA/probe mixtures were digested with RNAse A (Sigma) and RNAse T1 (Life Technologies). A mixture of 2.0 μg RNAse A and 1000 units of RNAse T1 in a buffer containing 330 mM NACl, 10 mM Tris (pH 8.0) and 5 mM EDTA (400 μl) was added to each sample and incubated for 60 min at room temperature. After digestion with RNAses, 20 μl of 10% SDS and 50 μg proteinase K were added to each tube and incubated at 37°C for 15 min. Samples were extracted with phenol/chloroform:isoamyl alcohol and precipitated in 2 volumes of ethanol for 1 hr at -70°C. Pellet Paint (Novagen) was added to each tube (2.0 μg) as a carrier to facilitate precipitation. Following precipitation, samples were centrifuged, washed with cold 70% ethanol, and vacuum dried. Samples were dissolved in formamide loading buffer and size-fractionated on a urea/acrylamide sequencing gel (7.0 M urea, 4.5% acrylamide in Tris-borate-EDTA). Gels were dried and apposed to storage phosphor screens and scanned using a phosphorimager (Molecular Dynamics).

RT-PCR

For the detection of low levels of RNA encoding hp-15a receptor, RT-PCR was carried out on mRNA extracted from human tissue. Reverse transcription and PCR reactions were carried out in 50 μl volumes using EzrTth DNA polymerase. Primers with the following sequences were used:

RA hp15F24

ACCTCACACTGGCTGATCTCCTCT (Seq. I.D. No. 19)

RA hp15B1

GTAGATGCCCATGAGGATGGTGGTG (Seq. I.D. No. 20)

Each reaction contained 0.2 μg mRNA and 0.3μM of each primer. Concentrations of reagents in each reaction were: 300μM each of dGTP, dATP, dCTP, dTTP; 2.5mM Mn(OAc)$_2$; 50mM Bicine; 115 mM K acetate, 8% glycerol and

5   5 units EzrTth DNA polymerase. All reagents for PCR (except mRNA and oligonucleotide primers) were obtained from Perkin Elmer. Reactions were carried out under the following conditions: 65°C 60 min, 94°C 2 min, (94°C 1 min, 65°C 1 min) 40 cycles, 72°C 10 min. PCR

10   reactions were size fractionated by agarose gel electrophoresis, DNA stained with ethidium bromide (EtBr) and photographed with UV illumination.

Positive controls for PCR reactions consisted of

15   amplification of the target sequence from a plasmid construct, as well as reverse transcribing and amplifying a known sequence. Negative controls consisted of mRNA blanks as well as primer blanks. To confirm that the mRNA was not contaminated with genomic

20   RNA, samples were digested with RNAses before reverse transcription. Integrity of RNA was assessed by amplification of mRNA coding for GAPDH.

Results and Discussion

A human genomic placenta library was screened, under reduced stringency conditions, with oligonucleotide probes directed to the third, sixth, and seventh transmembrane regions of what was later designated the human 5-HT$_{1D\beta}$ receptor (Weinshank, et al., 1992), and the human 5-HT$_{1A}$ receptor (Fargin, A., et al., 1988). Positively-hybridizing clones were isolated, plaque-purified and characterized by Southern blot analysis and sequencing. One clone, hp15a, contained a 223bp PstI fragment of hp15a DNA hybridizing with the TM III oligos. Sequence analysis revealed that the fragment encoded a novel GPCR-like TM III domain with an unusual predicted amino acid sequence motif, "LGRY", rather than the commonly observed "LDRY" sequence at that location.

In an attempt to obtain the entire coding region of the putative GPCR a ~3 kb BglII fragment that hybridized with the same probes was subcloned into pUC18 (designated K49). Sequence analysis showed that the fragment could encode TMs I through VII but not a starting methionine, indicating that the N-terminus was truncated. To obtain the full 5' coding region a ~750 bp BamHI/HindIII fragment of the genomic clone hp15a was subcloned and sequenced. Since this fragment contained an in-frame start codon and stop codons further upstream in all three reading frames, it appeared to encode the native N-terminus of the novel receptor. The BamHI/HindIII fragment was ligated with a HindIII/EcoRI fragment of the previously described BglII fragment into pUC18 for subsequent isolation of a BamHI/EcoRI fragment encoding the complete coding region. This fragment was blunted and ligated into the expression vector pcEXV-3; a single colony containing the full-length hp15a DNA in the correct orientation (designated K90) was selected for further analysis.

The longest open reading frame in this construct, K90, is predicted to encode a protein of 396 amino acids with only one potential initiating methionine. Hydropathy analysis of the protein is consistent with a putative topography of seven transmembrane domains (data not shown), indicative of the G protein-coupled receptor family.

Other features of this human hp15a receptor gene are the presence of 2 potential sites for N-linked glycosylation in the amino terminus (asparagine residues 3 and 8) and the presence of several serines and threonines in the carboxyl terminus and intracellular loops, which may serve as sites for potential phosphorylation by protein kinases.

Localization

Detection of mRNA coding for hp15a: Human mRNA was isolated and assayed as described from: liver, kidney, lung, heart, stomach, small intestine, spleen, pancreas, placenta, striated muscle, pituitary and CNS regions. CNS regions included: whole brain, amygdala, hippocampus, spinal cord, cerebellum, thalamus, substantia nigra, and caudate. Fetal tissue was obtained from a 25 week fetus and included: brain, liver, lung, and kidney. The distribution of mRNA encoding hp15a is widespread with the highest levels found in lung, spinal cord, and fetal lung, fetal liver and fetal kidney. Lower amounts are found broadly distributed as indicated in Table 1.

Table 1

Distribution of mRNA coding for hp15a receptor

Region	hp15a	Potential Applications
liver	-	Diabetes
kidney	-	Hypertension, Electrolyte balance
lung	++	Respiratory disorders, asthma
heart	-	Cardiovascular indications
stomach	-	Gastrointestinal disorders
small intestine	-	Gastrointestinal disorders
spleen	+	Immune function
pancreas	-	Diabetes, endocrine disorders
placenta	++	Gestational disorders
Striated muscle	-	Musculskeletal disorders
pituitary	+	Endocrine/neuroendocrine regulation
whole brain	+	
amygdala	+	Anxiolysis, Depression, Regulation of appetite, and Affective disorders
hippocampus	+	Cognitition/memory
spinal cord	++	Analgesia, sensory modulation and transmission
cerebellum	+	Motor coordination
thalamus	+	Sensory integration
substantia nigra	+	Modulation of dopaminergic function and motor coordination
caudate	+	Modulation of dopaminergic function
fetal brain	+	Developmental disorders
fetal lung	+++	Developmental disorders
fetal kidney	++	Developmental disorders
fetal liver	+++	Developmental disorders

A comparison of nucleotide and peptide sequences of the hp15a receptor gene and the hp15a receptor, respectively, with sequences contained in the

Genbank/EMBL databases reveals that the clone is most related to the rat alpha 1B adrenergic receptor (26% amino acid identity), followed by the human NPY/PYY/PP Y4 receptor (24% amino acid identity). Also related are the human dopamine D3 receptor and another orphan receptor designated GPR14 (22.5% amino acid identity). These levels of homology are lower than is typically seen for receptor subtypes, thus the hp15a receptor is unlikely to be an adrenergic, dopaminergic, or NPY receptor. Its similar level of identity to GPCRs of multiple subfamilies (biogenic amine and neuropeptide) indicates that the endogenous ligand could be from any class of molecules interacting with GPCRs. However, it is not yet possible to accurately predict the nature of the endogenous ligand from primary sequence alone. The cloning of the gene encoding the hp15a receptor has nevertheless provided the means to explore its physiological roles by pharmacological characterization, and by Northern and *in situ* mapping of its mRNA distribution. Further, the availability of the DNA encoding the hp15a receptor will facilitate the development of antibodies and antisense technologies useful in defining the functions of the gene product *in vivo*. Antisense oligonucleotides which target mRNA molecules to selectively block translation of the gene product *in vivo* have been used successfully to relate the expression of a single gene with its functional sequelae. The cloning of the hp15a receptor gene will allow the use of this approach to explore the functional consequences of blocking the expression of its mRNA without knowledge of its endogenous ligand. Thus, the cloning of this receptor gene provides the means to explore its physiological roles in the nervous system and elsewhere, and may thereby help to elucidate structure/function relationships within the GPCR superfamily.

In conclusion, the primary structure of the protein encoded by the hp15a receptor gene and its lack of close identity with existing GPCRs indicate that the endogenous ligand may represent any class of neuroregulatory substances, and further suggest that additional members of this new receptor subfamily may exist.

REFERENCES

U.S. Patent No. 5,602,024 (Gerald et al. Feb. 11, 1997).

5 Coleman, A. (1984) Transcription and Translation: A Practical Approach (B.D. Hanes, S.J. Higgins, eds., pp 271-302, IRL Press, Oxford, 1984)

Dascal, N., Schreibmayer, W., Lim, N. F., Wang, W.,
10 Chavkin, C., DiMagno, L., Labarca, C., Kieffer, B.L., Gaveriaux-Ruff, C., Trollinger, D., Lester, H. A., Davidson, N. (1993) Proc. Natl. Acad. Sci. USA 90:10235-10239.

15 Fargin, A.; Raymond, J. R.; Lohse, M. J.; Kobilka, B. K.; Caron, M. G.; Lefkowitz, R. J. Nature 335:358-360 (1988).

Fong, T. M., Huang, R. C., Yu, H., Swain, C. J.,
20 Underwood, D., Cascieri, M. A., and Strader, C.D., Can. J. Physiol. Pharmol. 73(7):860-865 (Jul 1995).

Graziano, M.P.; Hey, P.J., Strader, C.D., Receptors Channels 4(1):9-17 (1996).
25
Guam, X.M., Amend, A., Strader, C.D., Mol. Pharmacol. 48(3):492-498 (Sep 1995).

Krapivinsky, G., Gordon, E. A., Wickman B.,
30 Velimirovic, B., Krapivinsky, L., Clapham, D. E. (1995) Nature 374:135-141.

Krapivinsky, G., Krapivinsky, L., Velimirovic, B., Wickman, K., Navarro, B., Clapham, D. E., (1995b) J.
35 Biol. Chem. 270:28777-28779.

Kubo, Y., Reuveny, E., Slesinger, P. A., Jan, Y. N.,

Jan, L.Y. (1993) Nature 364:802-806.

Masu, Y. et al. (1994) Nature 329:21583-21586.

5   Miller, J., Germain, R.N., Efficient cell surface
    expression of class II MHC molecules in the absence of
    associated invariant chain. J.Exp.Med. 164:1478-1489
    (1986).

10 Sambrook, J., Fritsch, E. F., and Maniatis, T., In:
    Molecular Cloning: A Laboratory Manual, 2nd Edition
    (Cold Spring Harbor Laboratory, Cold Spring Harbor,
    N.Y.), 1989.

15 Sanger, F., Nicklen, S. and Coulsen, A. R. Proc. Natl.
    Acad. Sci. USA 74:5463-5467 (1977).

    Southern, E. M. Detection of specific sequences among
    DNA fragments separated by gel electrophoresis. J.
20 Mol. Biol. 98:503-517 (1975).

    Spurney, R.F., Coffman, T.M. J. Pharmacol. Exp. Ther.
    283(1):207-215 (Oct. 1997).

25 Weinshank, R. L.; Zgombick, J. M.; Macchi, M. J.;
    Branchek, T. A.; Hartig, P. R. Proc. Natl. Acad. Sci.
    U.S.A. 89(8):3630-3634 (1992).

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Smith, Kelli

    (ii) TITLE OF INVENTION: DNA Encoding A Human Receptor (hp15a)
        And Uses Thereof

  (iii) NUMBER OF SEQUENCES: 2

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    (v) COMPUTER READABLE FORM:
      (A) MEDIUM TYPE: Floppy disk
      (B) COMPUTER: IBM PC compatible
      (C) OPERATING SYSTEM: PC-DOS/MS-DOS
      (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

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      (A) APPLICATION NUMBER: US Not Yet Known
      (B) FILING DATE: Herewith
      (C) CLASSIFICATION:

  (viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 1311 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TATGTTGCAG TTAGCTGGGG GGTGGTGGTG GCTGTGACAG GCACCGTGGG CAATGTGCTC 180

ACCCTACTGG CCTTGGCCAT CCAGCCCAAG CTCCGTACCC GATTCAACCT GCTCATAGCC 240

AACCTCACAC TGGCTGATCT CCTCTACTGC ACGCTCCTTC AGCCCTTCTC TGTGGACACC 300

TACCTCCACC TGCACTGGCG CACCGGTGCC ACCTTCTGCA GGGTATTTGG GCTCCTCCTT 360

TTTGCCTCCA ATTCTGTCTC CATCCTGACC CTCTGCCTCA TCGCACTGGG ACGCTACCTC 420

CTCATTGCCC ACCCTAAGCT TTTTCCCCAA GTTTTCAGTG CCAAGGGGAT AGTGCTGGCA 480

CTGGTGAGCA CCTGGGTTGT GGGCGTGGCC AGCTTTGCTC CCCTCTGGCC TATTTATATC 540

CTGGTACCTG TAGTCTGCAC CTGCAGCTTT GACCGCATCC GAGGCCGGCC TTACACCACC 600

ATCCTCATGG GCATCTACTT TGTGCTTGGG CTCAGCAGTG TTGGCATCTT CTATTGCCTC 660

ATCCACCGCC AGGTCAAACG AGCAGCACAG GCACTGGACC AATACAAGTT GCGACAGGCA 720

AGCATCCACT CCAACCATGT GGCCAGGACT GATGAGGCCA TGCCTGGTCG TTTCCAGGAG 780

CTGGACAGCA GGTTAGCATC AGGAGGACCC AGTGAGGGGA TTTCATCTGA GCCAGTCAGT 840

GCTGCCACCA CCCAGACCCT GGAAGGGGAC TCATCAGAAG TGGGAGACCA GATCAACAGC 900

AAGAGAGCTA AGCAGATGGC AGAGAAAGC CCTCCAGAAG CATCTGCCAA AGCCCAGCCA 960

ATTAAAGGAG CCAGAAGAGC TCCGGATTCT TCATCGGAAT TGGGAAGGT GACTCGAATG 1020

TGTTTTGCTG TGTTCCTCTG CTTTGCCCTG AGCTACATCC CCTTCTTGCT GCTCAACATT 1080

CTGGATGCCA GAGTCCAGGC TCCCCGGGTG GTCCACATGC TTGCTGCCAA CCTCACCTGG 1140

CTCAATGGTT GCATCAACCC TGTGCTCTAT GCAGCCATGA ACCGCCAATT CCGCCAAGCA 1200

TATGGCTCCA TTTTAAAAAG AGGGCCCCGG AGTTTCCATA GGCTCCATTA GAACTGTGAC 1260

CCTAGTCACC AGAATTCAGG ACTGTCTCCT CCAGGACCAA AGTGGCAGGT A 1311

(2) INFORMATION FOR SEQ ID NO:2:

      (i) SEQUENCE CHARACTERISTICS:
         (A) LENGTH: 396 amino acids
         (B) TYPE: amino acid
         (C) STRANDEDNESS: single
         (D) TOPOLOGY: linear

     (ii) MOLECULE TYPE: protein

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Leu Gly Tyr Arg Tyr Val Ala Val Ser Trp Gly Val Val Val Ala Val
            20                  25                  30

Thr Gly Thr Val Gly Asn Val Leu Thr Leu Leu Ala Leu Ala Ile Gln
            35                  40                  45

Pro Lys Leu Arg Thr Arg Phe Asn Leu Leu Ile Ala Asn Leu Thr Leu
    50                  55                  60

Ala Asp Leu Leu Tyr Cys Thr Leu Leu Gln Pro Phe Ser Val Asp Thr
65                  70                  75                      80

Tyr Leu His Leu His Trp Arg Thr Gly Ala Thr Phe Cys Arg Val Phe
            85                  90                  95

Gly Leu Leu Leu Phe Ala Ser Asn Ser Val Ser Ile Leu Thr Leu Cys
            100                 105                 110

Leu Ile Ala Leu Gly Arg Tyr Leu Leu Ile Ala His Pro Lys Leu Phe
            115                 120                 125

Pro Gln Val Phe Ser Ala Lys Gly Ile Val Leu Ala Leu Val Ser Thr
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Trp Val Val Gly Val Ala Ser Phe Ala Pro Leu Trp Pro Ile Tyr Ile
145                 150                 155                     160

Leu Val Pro Val Val Cys Thr Cys Ser Phe Asp Arg Ile Arg Gly Arg
            165                 170                 175

Pro Tyr Thr Thr Ile Leu Met Gly Ile Tyr Phe Val Leu Gly Leu Ser
            180                 185                 190

Ser Val Gly Ile Phe Tyr Cys Leu Ile His Arg Gln Val Lys Arg Ala
            195                 200                 205

Ala Gln Ala Leu Asp Gln Tyr Lys Leu Arg Gln Ala Ser Ile His Ser
    210                 215                 220

Asn His Val Ala Arg Thr Asp Glu Ala Met Pro Gly Arg Phe Gln Glu
225                 230                 235                     240

Leu Asp Ser Arg Leu Ala Ser Gly Gly Pro Ser Glu Gly Ile Ser Ser
            245                 250                 255

Glu Pro Val Ser Ala Ala Thr Thr Gln Thr Leu Glu Gly Asp Ser Ser
            260                 265                 270

Glu Val Gly Asp Gln Ile Asn Ser Lys Arg Ala Lys Gln Met Ala Glu
            275                 280                 285

Lys Ser Pro Pro Glu Ala Ser Ala Lys Ala Gln Pro Ile Lys Gly Ala
    290                 295                 300
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Arg Arg Ala Pro Asp Ser Ser Ser Glu Phe Gly Lys Val Thr Arg Met
305   310   315   320

Cys Phe Ala Val Phe Leu Cys Phe Ala Leu Ser Tyr Ile Pro Phe Leu
             325   330   335

Leu Leu Asn Ile Leu Asp Ala Arg Val Gln Ala Pro Arg Val Val His
             340   345   350

Met Leu Ala Ala Asn Leu Thr Trp Leu Asn Gly Cys Ile Asn Pro Val
     355   360   365

Leu Tyr Ala Ala Met Asn Arg Gln Phe Arg Gln Ala Tyr Gly Ser Ile
     370   375   380

Leu Lys Arg Gly Pro Arg Ser Phe His Arg Leu His
385   390   395